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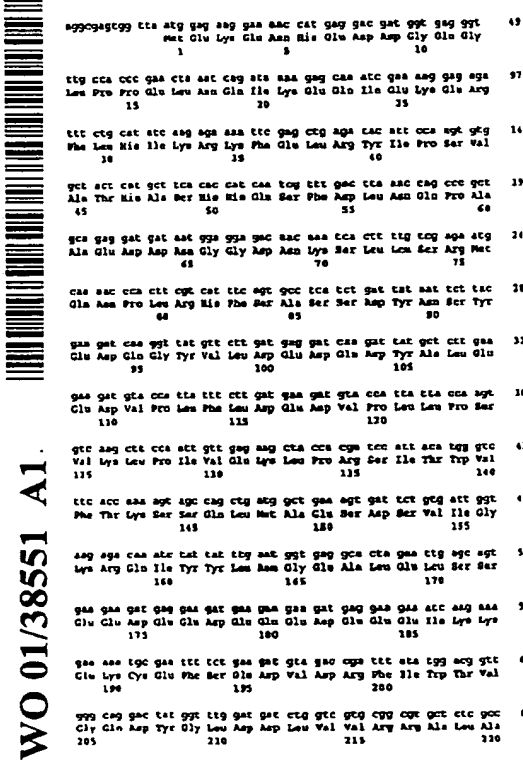
(54) Title: REGULATION OF POLYCOMB GROUP GENE EXPRESSION FOR INCREASING SEED SIZE IN PLANTS



(57) Abstract: The present invention relates to regulation of Polycomb group gene expression to provide for larger seed size by plants. Applicants have discovered that an absence of seed specific Polycomb group gene expression in early seed development (from fertilization to late heart, torpedo or cellularization stage) and expression of the gene in late seed development, (any time after these developmental states), yields larger seeds. Methods and compositions for such manipulation are disclosed.

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TITLE: REGULATION OF POLYCOMB GROUP GENE EXPRESSION FOR INCREASING SEED SIZE IN PLANTS

FIELD OF THE INVENTION

This invention relates to a method of increasing seed size in plants. Specifically, the invention relates to the functional regulation of Polycomb group gene expression to provide for an increase in seed size over those seeds without such regulatory manipulation.

BACKGROUND OF THE INVENTION

Polycomb group genes regulate the expression of homeotic genes as well as cellular proliferation. In the development of multicellular organisms, the process of determination requires the maintenance of specific patterns of gene expression through many cycles of cell division.

Homeotic genes specify the identity of serially repeated units such as the whorls of flowers or the segments of insects (Coch, E.S., et al., "The war of the whorla genetic interactions controlling flower development", Nature, 353:31-37 (1991); Lewis, "A gene complex controlling segmentation in *Drosophila*, Nature 276:565-570 (1978)). In both plants and animals they are transcribed in precise spatial patterns, often throughout development, and the particular combination of genes active in a cell specifies its identity. In several cases, expression late in development is both necessary and sufficient to specify identity (Bowman, J.L., "Genes directing flower development in Arabidopsis", Plant Cell, 1, 37-52 (1989; Carpenter, R, et al., "Floral homeotic mutations produced by transposon-mutagenesis in ...", Genes Dev., 4:1483-1493 (1990); Struhl, G., "Genes controlling segmentation specification in the Drosophila thorax", Proc. Natl Acad. Sci. USA, 79:7360-7384 (1982)). Because homeotic gene expression patterns are specified early in development as a result of complex genetic interactions (reviewed in refs Ingham, P.W., "The molecular genetics of embryonic pattern formation in Drosophila", Nature, 335:25-33 (1988); Weigel, "The ABCs of doral homeotic genes", Cell, 78:203-209 (1994)), this raises the problem of how these patterns are faithfully maintained and propagated through cell divisions during later development.

In *Drosophila* embryogenesis, regional fate becomes determined early, at around the cellular blastoderm stage (Simons, A., "When does determination occur in Drosophila embryos", Dev. Biol., 97:212-221 (1983)), and alternative fates are selected and determined by the persistent activity of homeotic genes (Lawrence, P.A., IN Insect Development, ed. Lawrence, P.A. 132-148 (Blackwell, Oxford, 1976)). The maintenance of expression boundaries during later stages of development requires the activity of two antagonistic groups of genes, the Polycomb-group (Pc-G) and the trithorax-group (trx-G) (Simon, J., "Locking in stable states of gene expression: transcriptional control during

Drosophila development", Curr. Opin. Cell Hint., 7:376-385 (1995); Kennison, J.A., "Transcriptional activation of Drosophila homeotic genes from distant regulatory elements", Trends Genec. 9:75-79 (1993)).

Polycomb group (Pc-G) genes and *trithorax* group in *Drosophila* encode transacting factors that are responsible for preventing the transcription of homeotic selector (HOM-C) genes outside of their appropriate expression domains through positive and negative regulation (Duncan and Lewis, 1982; Struhl and Akam, 1985; Paro, 1990). There are several criteria according to which genes are classified as members of the Polycomb group. Loss-of-function mutations in Pc-G genes produce phenotypes similar to those caused by loss of Polycomb function. In embryos, this results in the disruption of the anterior/posterior expression boundaries of HOM-C genes of the ANTP-C and BX-C leading to ectopic expression of *abd-A* and *Abd-B* (Simon et al., 1992) and transformation of the thoracic and abdominal segments to the identity of the eighth abdominal segment.

Enhancer of zeste (E*(z)*) has been classified as a Pc-G gene (Jones and Gelbart, 1990; Phillips and Shearn, (1990). A loss-of-function allele of the E(z) gene was recovered in a screen for late larval/pupal recessive lethal mutations that cause imaginal disc abnormalities (Shearn et al., 1971). It also however exhibits several properties of the *trithorax* group as well (LaJeunesse and Shearn 1996).

In plants, the role of homeotic genes is best understood with respect to flower development. Flowers typically contain four concentric whorls of organs with identity sepal (whorl 1), petal (whorl 2), stamen (whorl 3) and carpel (whorl 4). Based on the genetic and morphological analysis of floral homeotic mutants, a model has been proposed to account for the specification of organ identity in the different whorls based on combinatorial action of homeotic genes (Coch, E.S., et al., "The war of the whorla genetic interactions controlling flower development", Nature, 353:31-37 (1991)). In *Arabidopsis*, the homeotic AGAMOUS (AG) gene is required to specify stamen and carpal identity in whorls 3 and 4 respectively. Molecular isolation of the AG gene indicates that it encodes a protein belonging to the MADS box family of transcription factors, and that its RNA is confined to its domain of function in whorls 3 and 4 (Yanofsky, M.F., et al., "The protein encoded by the Arabidopsis homeotic gene AGAMOUS resembles transcription factors", Nature, 346:35-39 (1990); Drews, G.N., "Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product", Cell, 63: 991-1002 (1991)).

The plant life cycle alternates between a diploid and a haploid generation, the sporophyte and the gametophyte. Unlike in animals where meiotic products differentiate directly into gametes, the plant spores undergo several divisions to form a multicellular organism. Differentiation of the gametes occurs later in gametophyte development.

In most flowering plants, the mature female gametophyte consists of seven cells: three antipodals, two synergids, the egg cell, and a binucleate central cell whose nuclei fuse prior to fertilization The delivery of two sperm cells into the multicellular female gametophyte ensures fertilization of both the egg cell and the binucleate central cell, the precursors of the embryo and the triploid endosperm, respectively. Viable seed formation depends on the coordinated development of the embryo, the endosperm, and the maternal seed coat. Although these interactions are poorly understood, seed morphogenesis requires maternal gene activity in the haploid as well as in the diploid tissues of the developing ovule.

It has previously been shown that A seed specific Polycomb group gene exists which has been isolated and purified from *Arabidopsis thaliana. The seed specific Polycomb group gene MEDEA* (*MEA*) regulates cell proliferation by exerting a gametophytic maternal control during seed development. Grossniklaus et al. (1998), Maternal control of embryogenesis by *MEDEA*, a *Polycomb* group gene in *Arabidopsis*. *Science* 280:446-450. It also been found that seeds derived from embryo sacs carrying a mutant *mea*-1 allele (hereinafter referred to as *mea* seeds) abort after delayed morphogenesis with excessive cell proliferation in the embryo and reduced free nuclear divisions in the endosperm. The *mea* mutation affects an imprinted gene expressed maternally in cells of the female gametophyte and after fertilization only from maternally inherited *MEA* (wild-type) alleles.

Five alleles of *mea* have been described, all of which are likely to be recessive loss-of-function mutations. *MEA* encodes a SET domain protein with homology to members of the *Polycomb* and *trithorax* group, which are believed to maintain active or repressed states of gene expression during development by modulating higher order chromatin structure. (Kennison, 1995; Orlando and Paro, 1995; Pirotta, 1997). The nature of the maternal effect on seed development in *mea* mutants is not completely understood. Because the endosperm inherits two maternal copies but only one paternal copy of the genome, it is believed that *mea* could affect a dosage-sensitive gene required for endosperm development. Alternatively, the mutation could disrupt a maternally produced gene product stored in the egg and/or central cell, which is subsequently required for seed development. As a third possibility, the mutation could affect an imprinted gene that is transcribed exclusively from the maternally inherited alleles after fertilization.

As can be seen a need exists in the art for methods and compositions which regulate Polycomb group expression. Knowledge of regulation and concomitant effects on Polycomb expression will allow breeders to increase seed size, control timing seed set, or develop varieties completely devoid of seed.

As can be seen there is a continuing need in the art for methods and techniques to manipulate seed development to provide for phenotypes, such as seed size, which will be of value to plant breeders and to farmers.

It is therefore an object of the present invention to provide for regulation of Polycomb group genes to create plants with large seed size.

It is yet another object of the invention to provide expression constructs, sequences and transformed cells which provide for the manipulation of plant cells to induce production of the phenotype of larger seed size.

It is yet another object of the invention to provide for transgenic as well as nontransgenic (screening for naturally occurring or induced mutants)to provide for mechanisms to manipulate seed development.

It is yet another object of the invention to provide breeding materials that may be used in a breeding program to produce commercially crops with larger seed size.

These and other objects will become apparent from the following detailed description of the invention.

SUMMARY OF THE INVENTION

The present invention relates to the spatial and/or temporal regulation of Polycomb group gene expression to provide for larger seed production by plants. According to the invention, the timing of seed specific Polycomb group gene expression is critical and may be manipulated according to the teachings herein.

Applicants have discovered that an absence of seed specific Polycomb group gene expression in early seed development (from fertilization to late heart, torpedo or cellularization stage) and expression of the gene in late seed development, (any time after these developmental states), yields larger seeds.

Thus the invention comprises manipulation of seed specific Polycomb group gene expression such that wild-type gene expression is absent, inhibited, or suppressed during early seed development or so that the gene product is inactivated, during this period. Active gene product then is allowed to occur in late seed development. The scheme is summarized in the following table:

early seed development	no active Polycomb group gene product	(GENE OFF)or protein inactive
late seed development	Active Polycomb group gene product	(GENE ON)or protein active

Gene expression can be manipulated to any of a number of techniques well known to those of skill in the art and described in detail herein and in the materials incorporated by

reference, including but not limited to gene activation by use of tissue specific, time specific or inducible promoters with an appropriate transgenic expression system; or inactivation to manipulate Polycomb group gene expression: such as screening for identification or inactive mutants, or transgenic protocols such as inactivation by mutation, expression of a second site modifier gene, sense co-suppression anti-sense co-suppression, homologous recombination, or double stranded RNA interference (dsRNA).

In one embodiment of the invention, applicants have demonstrated the ability to manipulate a Polycomb group gene's expression by using a second site mutation which removes the imprint at the paternally inherited Polycomb allele.

A mutation in the MEA modifier gene has been identified which is a regulator of genomic imprinting which can be used to regulate Polycomb expression to produce larger seed size in plants. This mutant modifier gene, *DDM1*, is required for the maintenance, but not the establishment, of the imprint at the *mea* locus in *Arabidopsis*. Further, mutations at the *decrease in DNA methylation1* (*ddm*1) locus are able to rescue *mea* seeds during seed development. Quite unexpectedly, these rescued seeds have the desirable trait of being larger than the wild type seeds.

Larger seeds provide the benefit of ensuring a better start for their seedlings since they contain larger amounts of stored food, as well as providing ease of handling for the breeder. Larger seeds also provide benefits in that the seed storage proteins are produced in greater numbers providing improved nutrition for feed, and for harvesting of important native or transgenic proteins from seeds. Thus, applicants have identified a critical step in the regulation of Polycomb group genes that can be manipulated to induce larger seeds in plants.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1 is the MEA 1 wild type sequence.

Figures 2A-2J are photographs depicting the localization of *MEA* mRNA in ovules and developing seeds of wild-type *Arabidopsis*. Magnification: bar is 17 μm in (A); 22 μm in (B); 4.3 μm in (C); 35 μm in (D) to (G); 51 μm in (H) to (J).

Figure 2A is an ovule (ov) containing an 8-nucleated non-cellularized female gametophyte (fg). The *MEA* transcript is present in the developing female gametophyte.

Figure 2B is a mature ovule with unfertilized female gametophyte; *MEA* mRNA is present in the cytoplasm of the synergids (sy), the egg cell (ec), and the central cell containing a homo-diploid fused polar nucleus (fpn).

Figure 2C is detail of the synergids (sy) and the fused polar nucleus (fpn) shown in (B); the transcript is localized in the cytoplasm of the synergids, but appears closely associated with the fused polar nucleus.

Figure 2D is *MEA* mRNA localized in the globular embryo (e) and in the free nuclear endosperm (fne) of a developing seed; artifactual staining in the endothelium (en) is seen in sense and anti-sense experiments.

Figure 2E is a globular embryo hybridized with a sense *MEA* probe.

Figure 2F is a heart stage embryo hybridized with an anti-sense probe; the *MEA* transcript is localized in the embryo(e) and the free nuclear endosperm (fne).

Figure 2G is a heart stage embryo hybridized with a sense *MEA* probe.

Figure 2H is a developing seed with early torpedo embryo (e), cellularized endosperm, and seed coat (sc); *MEA* mRNA is absent from the cellularized endosperm.

Figure 2I is a developing seed with cotyledonary embryo.

Figure 2J is a developing seed showing cellularized endosperm (ce), and the seed coat (sc); *MEA* mRNA is localized in the free endosperm (fne).

Figure 3 are photographs depicting the segregation of mea alleles in a duplex tetraploid. In tetraploids, the frequencies of the different gamete classes depend on the coefficient of double reduction, c, which is the frequency at which the alleles of two sister chromatids are recovered in the same gamete. Small boxes represent haploid genomes from mutant *mea*-1 (red) or wild-type *MEA* (yellow) alleles. For the chromosomal region containing the mea locus, c has been estimated as c = 0.1. In duplex tetraploids, the frequencies of *MEA*/*MEA*, *MEA*/*mea*-1, and *mea*-1/*mea*-1 gametes are (1+2c)/6 = 0.2, 4(1-c)/6/ = 0.6, and (1+2c)/6 = 0.2, respectively. These values were used to calculate the frequencies of the different endosperm classes. If the maternal genotype determines the seed phenotype independent of gene dosage, the following endosperm genotypes are expected to develop normally (m, maternal; p, paternal): 4m *MEA*/2p *MEA*; 4m *MEA*/1p *MEA*, 1p *mea-1*; 4m *MEA*/2p *mea-1*; 2m *MEA*, 2m *mea-1*/2p *MEA*; 2m *MEA*, 2m *mea-1*/1p *MEA*, 1p *mea-1*; and 2m *MEA*, 2m *mea-1*/2p *mea-1* amounting to 78.8% if a spontaneous seed abortion rate of 1.5% as determined for the parental tetraploid is included [(0.04+0.12+0.04+0.12+0.36+0.12)*(100-1.5)% = 78.8%]. If normal seed development depends on an excess of wild-type *MEA* product independent of parental origin in the endosperm (*MEA*:*mea*-1>3:3) we expect the following endosperm genotypes to develop normally: 4m *MEA*/2p *MEA*; 4m *MEA*/1p *MEA*, 1p *mea-1*; 4m *MEA*/2p *mea-1*; 2m *MEA*, 2m *mea-1*/2p *MEA* amounting to 31.5% [0.12+0.04+0.12+0.04)*(100-1.5)% = 31.52%].

Figures 4A-4E are photographs depicting MEA transcription in the central cell before and after fertilization. Magnification: bar is approximately 0.5 μm in (A); 1.2 μm in (B); 1.4 μm in (C); 1.5 μm in (D); 1.7 μm in (E).

Figure 4A are two haploid polar nuclei in the unfertilized central cell; a single nuclear dot is localized in each nucleus.

Figure 4B is a tetraploid nucleus (resulting from the fusion of two diploid polar nuclei in a tetraploid ovule) showing four nuclear dots prior to fertilization.

Figure 4C is a diploid polar nucleus in the central cell of a diploid ovule prior to fertilization showing two nuclear dots.

Figure 4D is a triploid nuclei resulting from the division of the primary endosperm nucleus, each showing two nuclear dots after fertilization.

Figure 4E are two nuclear dots in the triploid endosperm nuclei located at the chalazal pole of the embryo sac.

Figure 5 is photograph depicting the expression of the mea-1 allele during early seed development. The two panels show amplification of *mea-1* and *ACTIN-11* (*ACT11*) as a control for cDNA synthesis. RNA was isolated from siliques derived from self-pollinated *mea-1* homozygous pistils (mea x mea), self-pollinated wild-type pistils (wt x wt), a cross between a homozygous mea-1 female and wild-type male (*mea* x wt), and a cross between wild-type female and homozygous *mea*-1 male (wt x *mea*). Primers that specifically amplify the *mea*-1 allele under these conditions were used for RT-PCR. (M) indicates the marker lane and (G) genomic DNA as a control.

Figure 6 is photograph depicting size comparison of *mea-1^m/MEA^p; ddm1-2/ddm1-2* and wild-type seeds and embryos. (A) Rescued *mea-1^m/MEA^p; ddm1-2/ddm1-2* (top) are larger than their sibling wild-type seeds (bottom). (B) The difference in size is also reflected in embryos from dissected from rescued (top) and wild-type seeds (bottom); scale bar = 400μm.

Figure 7 is a photograph depicting the morphology of *mea-1^m/MEA^p; ddm1-2/ddm1-2* seeds.

(A-D) Diagrams illustrating the three different classes of rescued seeds; (F-I) Morphological analysis of rescued embryos belonging to class depicted in (B). **(A)** Wild-type seed; **(B)** Rescued seed with giant embryo and partial endosperm; **(C)** Rescued seed with partially bent embryo and massive endosperm; **(D)** Rescued seed with T-shaped embryo and large volume of endosperm. **(E)** General morphology of a rescued seed belonging to class depicted in (B); the arrowhead indicates an abnormal region of cell proliferation in the hypocotyl. **(F)** Sagital section of a wild-type cotyledon. **(G)** Sagital section of a rescued seed cotyledon with small undifferentiated cells (arrowheads). **(H)** Detail of (E); Partially cellularized endosperm persisting at the chalazal region of a rescued seed. Abbreviations: CE= cellularized endosperm; Ep= epidermis. Maginification: scale bars: (A) to (E) = 100 μm; (F)= 20 μm; (G) and (H) = 28 μm.

DETAILED DESCRIPTION OF THE INVENTION

A *Polycomb* group gene (MEA) isolated from *Arabidopsis* has previously been identified and is involved in cell proliferation associated with seed development. See,

Grossniklaus, et al., 1998. "Maternal Control of Embryogenesis by MEDEA - a Polycomb group gene in Arabidopsis", Science, 280:446-450. If the *MEA* gene product is missing, embryos overproliferate and the endosperm underproliferates. Thus, the *MEA* gene and protein product can regulate proliferation both negatively and positively depending on the tissue. The wild type protein restricts embryo growth but promotes endosperm proliferation. A role in the control of cell proliferation either positively or negatively has also been shown for some of the animal homologs and this aspect of SET domain protein function appears to be conserved. (Simon, J., "Locking in stable states of gene expression: transcriptional control during *Drosophila* development", Curr Opin. Cell Biol. Vol. 7, 376-385(1995)). Studies now indicate that *MEA* is zygotically transcribed after fertilization during embryo and endosperm development.

MEA (SEQ ID NO:1, Figure 1) encodes a SET domain protein with homology to members of the *Polycomb* and *trithorax* group, which are believed to maintain active or repressed states of gene expression during development by modulating higher order chromatin structure. (Kennison, 1995; Orlando and Paro, 1995; Pirotta, 1997). Other mutants such as *fertilization-independent endosperm (fie)* and *fertilization-independent seed2 (fis2)* have been implicated in the control of seed development and also show a gametophytic maternal effect on seed formation and autonomous endosperm development. Ohad et al. (1999), Mutations in *FIE*, a WD Polycomb group gene, allow endosperm development without fertilization. *Plant Cell* 11:407-416. *FIE* encodes another member of the Polycomb group, whereas *FIS2* encodes a protein with a Zn-finger motif. Ming et al. (1999), Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 96:296-301.

Since the Polycomb group genes such as *MEA* gene are highly conserved in many other species, the maintenance of or inhibition of expression of such seed specific Polycomb group genes may be used to regulate the seed production size of many species other than *Arabidopsis*. *MEA* is a member of a large gene family in *Arabidopsis*. There are about 8 other SET domain proteins in the art. *MEA* is the only one with a known function in seed development, and is at the very least likely to be present in other angiosperm genomes, including the closely-related species *Brassica napus*, a species in which the seeds are used to obtain canola oil. This is particularly true since its structure and function is conserved even in animals.

The invention herein in its broadest sense contemplates the discovery that seed specific Polycomb group genes, one non-limiting example of which is MEA, can be regulated to provide for increased seed size in plants. According to the invention MEA gene activity must be absent or gene products produced must in inactive, during early seed

development (from fertilization to late heart or torpedo stage) and present during late seed development (after heart or torpedo stage).

This can be accomplished through any activation and/or inactivation techniques known to those of skill in the art such as use of tissue specific or inducible promoters in expression constructs containing the seed specific Polycomb gene product; or isolation of insertional mutations using reverse genetic screens, homologous recombination to generate loss of function alleles of a given gene, as well as transgenic protocols such as sense co-suppression, antisense co-suppression, and double stranded RNA interference (RNAi).

Antisense RNA has been used to inhibit plant target genes in a tissue-specific manner. van der Krol et al., Biotechniques 6:958-976 (1988). Antisense inhibition has been shown using the entire cDNA sequence as well as a partial cDNA sequence. Sheehy et al., Proc. Natl. Acad. Sci. USA 85:8805-8809 (1988); Cannon et al., Plant Mol. Biol. 15:39-47 (1990). There is also evidence that 3' non-coding sequence fragment and 5' coding sequence fragments, containing as few as 41 base-pairs of a 1.87 kb cDNA, can play important roles in antisense inhibition. (Chang et al., Proc. Natl. Acad. Sci. USA 86:10006-10010 (1989); Cannon et al., supra.

The phenomenon of co-suppression has also been used to inhibit plant target genes in a tissue-specific manner. Co-suppression of an endogenous gene using a full-length cDNA sequence as well as a partial cDNA sequence (730 bp of a 1770 bp cDNA) are known. Napoli et al., "The Plant Cell", Cell 2:291-299 (1990); Smith et al., Mol. Gen. Genetics 224:477-481 (1990).

Double stranded RNAi constructs produce a single transcript that encodes part of the gene to be silenced, a spacer, and the same part of the gene in antisense orientation. After transcription of this engineered gene, the RNA folds back on itself and forms a panhandle structure containing a piece of double stranded RNA. This method also works well if the spacer is an intron and in principle could get spliced out just leaving the dsRNA. See, Sharp, PA 1999, Genes and Development, "RNAi: and Double-Strand RNA", Jan 15; 13(2) pgs. 139-41.

Using a combination of these methods MEA or another homologous gene can be turned on or off in a predictable way; the same is true for its regulators such as ddm1. The state early MEA OFF, late MEA ON, can be achieved in a variety of ways using such general methods: A)expression of MEA or variants thereof under specific promoters, B)regulating expression of MEA by second site mutations such as ddm1 or C)chemically inducing MEA expression.

Under the concept of expression of MEA or variants thereof under specific promoters, protocols useful for the invention include: Expression of wild-type MEA activity in a mea mutant (or preferably a mea deficient) background caused by mutation of

gene silencing using a promoter that is specifically activated later during seed development only; expression of a gene silencing construct to inactivate MEA (e.g. sense, antisense or dsRNAi construct) under a promoter active only early during seed development; expression of wild-type MEA activity under an inducible promoter in a mea deficient background; (the inducing conditions are only applied late in seed development); expression of a gene silencing construct to inactivate MEA under an inducible promoter that is only induced early during seed development.

With respect to regulating expression of MEA by second site mutations such as ddm1, protocols useful for the invention include: in seeds inheriting an inactive mea allele from the mother, the paternally inherited wild-type MEA allele can be activated by a lack of activity of DDM1 (through mutation or gene silencing); other genes acting as second site modifiers will act similarly and are easily identified using routine screening techniques disclosed herein, ddm2, which is a mutation in the gene encoding DNA methyl transferase 1, has a similar effect;

With respect to chemically inducing MEA expression, protocols useful for the invention include: Drugs which demethylate DNA, for instance 5' azacytidine (5AC) and S-adenosyl-homocysteine (SAH). There are two modes of action through which DDM1 may act each of which can be manipulated through pharmacological drugs. Since ddm1 reduces DNA methylation levels to 30%, it is likely that the MEA gets reactivated by a change in its methylation pattern; Drugs known to affect chromatin organization, such as trichostatin A, sodium butyrate and others, a second mode of action is likely to go via changes in chromatin structure since DDM1 encodes a chromatin remodeling factor.

Due the highly conserved nature of the *MEA* gene product, it is expected that the gene or ones substantially equivalent thereto may be identified from other plants with similar seed specific functions. These homologs are intended to be within the scope of this invention. Similarly, the protein product disclosed here also many be used for other plants and many other muteins may be either engineered by those of skill in the art or isolated from other species. Homologous proteins or muteins as described herein and as isolated form other plants are also intended to be within the scope of this invention.

According to one embodiment of the invention, the *mea* mutation affects an imprinted gene expressed maternally in cells of the female gametophyte after fertilization only from maternally inherited *MEA* alleles, while paternally inherited MEA alleles are transcriptionally silent in both the embryo and the endosperm, at least early during seed development. Mutations at the *ddm1* locus are able to rescue *mea* seeds by functionally reactivating paternally inherited *MEA* alleles during seed development. Rescued seeds are larger than the wild type and show some of the abnormalities found in aborting *mea* seeds.

As used herein, the term "MEDEA" or "MEA" shall be interpreted to include any seed specific Polycomb group gene identified by the teachings herein or references incorporated herein and shall not be limited to any plant type. Such sequences will by substantially equivalent to the amino acid and nucleic acid sequence depicted in figure 1.

The term "substantially equivalent" as used herein means that the peptide is a substance having an amino acid with at least 30%-50% homology with at least one form of the protein as disclosed herein. 80% homology is preferred and 90% homology is most preferred especially including conservative substitutions. With respect to a nucleotide sequence the term substantially equivalent means that the sequence will encode a protein or peptide that is substantially equivalent.

Homology is calculated by standard methods which involve aligning two sequences to be compared so that maximum matching occurs, and calculating the percentage of matches. Substantially equivalent substances to these include those wherein one or more of the residues of the native sequence is deleted, substituted for, or inserted by a different amino acid or acids.

Preferred substitutions are those which are conservative, i.e., wherein a residue is replaced by another of the same general type. As is well understood, naturally occurring amino acids can be subclassified as acidic, basic, neutral and polar, or neutral and nonpolar. Furthermore, three of the encoded amino acids are aromatic. It is generally preferred that peptides differing from the native *MEA* sequence contain substitutions which are from the same group as that of the amino acid replaced. Thus, in general, the basic amino acids Lys and Arg are interchangeable; the acidic amino acids aspartic and glutamic are interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn are interchangeable; the nonpolar aliphatic acids Gly, Ala, Val, Ile, and Leu are conservative with respect to each other (but because of size, Gly and Ala are more closely related and Val, Ile and Leu are more closely related), and the aromatic amino acids Phe, Trp, and Tyr are interchangeable. While proline is a nonpolar neutral amino acid, it represents difficulties because of its effects on conformation, and substitutions by or for proline are not preferred, except when the same or similar conformational results can be obtained. Polar amino acids which represent conservative changes include Ser, Thr, Gln, Asn; and to a lesser extent, Met. In addition, although classified in different categories, Ala, Gly, and Ser seem to be interchangeable, and Cys additionally fits into this group, or may be classified with the polar neutral amino acids.

In general, whatever substitutions are made are such that the functional properties of the intact proteinaceous molecule is retained and ancillary properties, such as non-toxicity are not substantially disturbed.

Conditions of high stringency are those which will typically result in hybridization of sequences that are at least about 80% homologous as disclosed in Maniatis et al, Molecular Cloning, (1988) Cold Spring Harbor Press, Cold Spring Harbour, New York.

A "structural gene" is a DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

An "antisense oligonucleotide" is a molecule of at least 6 contiguous nucleotides, preferably complementary to DNA (antigene) or RNA (antisense), which interferes with the process of transcription or translation of endogenous proteins so that gene products are inhibited.

A "promoter" is a DNA sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' region of a gene, proximal to the transcriptional start site of a structural gene.

The term "expression" refers to biosynthesis of a gene product. Structural gene expression involves transcription of the structural gene into mRNA and then translation of the mRNA into one or more polypeptides.

The term "Co-suppression" is a method of inhibiting gene expression in plants wherein a construct is introduced to a plant. The construct has one or more copies of sequence which is identical to or which shares nucleotide homology with a resident gene.

"Homologous recombination" is another method of inhibiting gene function by introducing a disruption construct to a plant cell under conditions which facilitate recombination of endogenous genetic material with the construct.

A "cloning vector" is a DNA molecule such as a plasmid, cosmid, or bacterial phage that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance, kanamycin resistance, basta resistance, hygromycin resistance or ampicillin resistance.

An "expression vector" is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements including promoters, tissue specific regulatory elements, and enhancers. Such a gene is said to be "operably linked to" the regulatory elements.

A "recombinant host" may be any prokaryotic or eukaryotic cell that contains either a cloning vector or an expression vector. This term also includes those prokaryotic or

eukaryotic cells that have been genetically engineered to contain the clone genes in the chromosome or genome of the host cell.

A "transgenic plant" is a plant having one or more plant cells that contain an expression vector. Plant tissue includes differentiated and undifferentiated tissues or plants, including but not limited to roots, stems, shoots, leaves, pollen, seeds, tumor tissue, and various forms of cells and culture such as single cells, protoplasm, embryos, and callus tissue. The plant tissue may be in plant or in organ, tissue, or cell culture.

The invention in one aspect comprises expression constructs comprising a DNA sequence which encodes upon expression a seed specific *Polycomb* group gene product or *DDM1* regulatory gene product operably linked to a promoter to direct expression of the protein. These constructs are then introduced into plant cells using standard molecular biology techniques. The invention can be also be used for hybrid plant or seed production, once transgenic inbred parental lines have been established.

In another aspect the invention involves the inhibition of a *Polycomb* group gene product in plants through introduction of a construct designed to inhibit the same gene product. The design and introduction of such constructs based upon known DNA sequences is known in the art and includes such technologies as antisense RNA or DNA, co-suppression, double stranded RNA interference or any other such mechanism. Several of these mechanisms are described and disclosed in United States Patent 5,686,649 to Chua et. al, which is hereby expressly incorporated herein by reference.

The methods of the invention described herein may be applicable to any species of plant. Production of a genetically modified plant tissue either expressing or inhibiting expression of a structural gene combines the teachings of the present disclosure with a variety of techniques and expedients known in the art. In most instances, alternate expedients exist for each stage of the overall process. The choice of expedients depends on the variables such as the plasmid vector system chosen for the cloning and introduction of the recombinant DNA molecule, the plant species to be modified, the particular structural gene, promoter elements and upstream elements used. Persons skilled in the art are able to select and use appropriate alternatives to achieve functionality. Culture conditions for expressing desired structural genes and cultured cells are known in the art. Also as known in the art, a number of both monocotyledonous and dicotyledonous plant species are transformable and regenerable such that whole plants containing and expressing desired genes under regulatory control of the promoter molecules according to the invention may be obtained. As is known to those of skill in the art, expression in transformed plants may be tissue specific and/or specific to certain developmental stages. Truncated promoter selection and structural gene selection are other parameters which may be optimized to

achieve desired plant expression or inhibition as is known to those of skill in the art and taught herein.

The following is a non-limiting general overview of Molecular biology techniques which may be used in performing the methods of the invention.

PROMOTERS

The constructs, promoters or control systems used in the methods of the invention may include a tissue specific promoter, an inducible promoter or a constitutive promoter.

A large number of suitable promoter systems are available. For example one constitutive promoter useful for the invention is the cauliflower mosaic virus (CaMV) 35S. It has been shown to be highly active in many plant organs and during many stages of development when integrated into the genome of transgenic plants including tobacco and petunia, and has been shown to confer expression in protoplasts of both dicots and monocots.

Organ-specific promoters are also well known. For example, the E8 promoter is only transcriptionally activated during tomato fruit ripening, and can be used to target gene expression in ripening tomato fruit (Deikman and Fischer, EMBO J. (1988) 7:3315; Giovannoni et al., The Plant Cell (1989) 1:53). The activity of the E8 promoter is not limited to tomato fruit, but is thought to be compatible with any system wherein ethylene activates biological processes. Similarly the Lipoxegenase ("the LOX gene") is a fruit specific promoter.

Other fruit specific promoters are the 1.45 promoter fragment disclosed in Bird, et al., Plant Mol. Bio., pp 651-663(1988) and the polygalacturonase promoter from tomato disclosed in U.S. Patent 5,413,937 to Bridges et al.

Leaf specific promoters include as the AS-1 promoter disclosed in US Patent 5,256,558 to Coruzzi and the RBCS-3A promoter isolated from pea the RBCS-3A gene disclosed in US Patent 5,023,179 to Lam et al.

And finally root specific promoters include the Cam 35 S promoter disclosed in US Patent 391,725 to Coruzzi et al; the RB7 promoter disclosed in US patent 5,459,252 to Conking et al and the promoter isolated from Brassica napus disclosed in US Patent 5,401, 836 to Bazczynski et al. which give root specific expression.

Other examples of promoters include maternal tissue promoters such as seed coat, pericarp and ovule. Promoters highly expressed early in endosperm development are most effective in this application. Of particular interest is the promoter from the a' subunit of the soybean β-conglycinin gene [Walling et al., Proc. Natl. Acad. Sci. USA 83:2123-2127 (1986)] which is expressed early in seed development in the endosperm and the embryo.

Further seed specific promoters include the Napin promoter described in united States Patent 5,110,728 to Calgene, which describes and discloses the use of the napin

promoter in directing the expression to seed tissue of an acyl carrier protein to enhance seed oil production; the DC3 promoter from carrots which is early to mid embryo specific and is disclosed at Plant Physiology, Oct. 1992 100(2) p. 576-581, "Hormonal and Environmental Regulation of the Carrot Lea-class Gene Dc 3, and Plant Mol. Biol., April 1992, 18(6) p. 1049-1063, "Transcriptional Regulation of a Seed Specific Carrot Gene, DC 8": the phaseolin promoter described in United States Patent 5,504,200 to Mycogen which discloses the gene sequence and regulatory regions for phaseolin, a protein isolated from *P. vulgaris* which is expressed only while the seed is developing within the pod, and only in tissues involved in seed generation.

Other organ-specific promoters appropriate for a desired target organ can be isolated using known procedures. These control sequences are generally associated with genes uniquely expressed in the desired organ. In a typical higher plant, each organ has thousands of mRNAs that are absent from other organ systems (reviewed in Goldberg, Phil, Trans. R. Soc. London (1986) B314-343. mRNAs are first isolated to obtain suitable probes for retrieval of the appropriate genomic sequence which retains the presence of the natively associated control sequences. An example of the use of techniques to obtain the cDNA associated with mRNA specific to avocado fruit is found in Christoffersen et al., Plant Molecular Biology (1984) 3:385. Briefly, mRNA was isolated from ripening avocado fruit and used to make a cDNA library. Clones in the library were identified that hybridized with labeled RNA isolated from ripening avocado fruit, but that did not hybridize with labeled RNAs isolated from unripe avocado fruit. Many of these clones represent mRNAs encoded by genes that are transcriptionally activated at the onset of avocado fruit ripening.

Another very important method that can be used to identify cell type specific promoters that allow even to identification of genes expressed in a single cell is enhancer detection (O'Kane, C., and Gehring, W.J. (1987), "Detection *in situ* of genomic regulatory elements in *Drosophila*", Proc. Natl. Acad. Sci. USA, 84, 9123-9127). This method was first developed in *Drosophila* and rapidly adapted to mice and plants (Wilson, C., Pearson, R.K., Bellen, H.J., O'Kane, C.J., Grossniklaus, U., and Gehring, W.J. (1989), "P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*", Genes & Dev., 3, 1301-1313; Skarnes, W.C. (1990), "Entrapment vectors: a new tool for mammalian genetics", Biotechnology, 8, 827-831; Topping, J.F., Wei, W., and Lindsey, K. (1991), "Functional tagging of regulatory elements in the plant genome", Development, 112, 1009-1019; Sundaresan, V., Springer, P.S., Volpe, T., Haward, S., Jones, J.D.G., Dean, C., Ma, H., and Martienssen, R.A., (1995), "Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements", Genes & Dev., 9, 1797-1810).

The promoter used in the method of the invention may be an inducible promoter. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of a DNA sequence in response to an inducer. In the absence of an inducer, the DNA sequence will not be transcribed. Typically, the protein factor that binds specifically to an inducible promoter to activate transcription is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer may be a chemical agent such as a protein, metabolite (sugar, alcohol etc.), a growth regulator, herbicide, or a phenolic compound or a physiological stress imposed directly by heat, salt, toxic elements etc. or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell such as by spraying, watering, heating, or similar methods. Examples of inducible promoters include the inducible 70 kd heat shock promoter of D. melanogaster (Freeling, M., Bennet, D.C., Maize ADN 1, Ann. Rev. of Genetics, 19:297-323) and the alcohol dehydrogenase promoter which is induced by ethanol (Nagao, R.T., et al., Miflin, B.J., Ed. Oxford Surveys of Plant Molecular and Cell Biology, Vol. 3, p. 384-438, Oxford University Press, Oxford 1986) or the Lex A promoter which is triggered with chemical treatment and is available through Ligand pharmaceuticals. The inducible promoter may be in an induced state throughout seed formation or at least for a period which corresponds to the transcription of the DNA sequence of the recombinant DNA molecule(s).

Another example of an inducible promoter is the chemically inducible gene promoter sequence isolated from a 27 kd subunit of the maize glutathione-S-transferase (GST II) gene. Two of the inducers for this promoter are N,N-diallyl-2,2-dichloroacetamide (common name: dichloramid) or benzyl-=2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate (common name: flurazole). In addition, a number of other potential inducers may be used with this promoter as described in published PCT Application No. PCT/GB90/00110 by ICI.

Another example of an inducible promoter is the light inducible chlorophyll a/b binding protein (CAB) promoter, also described in published PCT Application No. PCT/GB90/00110 by ICI.

Inducible promoters have also been described in published Application No. EP89/103888.7 by Ciba-Geigy. In this application, a number of inducible promoters are identified, including the PR protein genes, especially the tobacco PR protein genes, such as PR-1a, PR-1b, PR-1c, PR-1, PR-A, PR-S, the cucumber chitinase gene, and the acidic and basic tobacco beta-1,3-glucanase genes. There are numerous potential inducers for these promoters, as described in Application No. EP89/103888.7.

The preferred promoters may be used in conjunction with naturally occurring flanking coding or transcribed sequences of the seed specific Polycomb genes or with any other coding or transcribed sequence that is critical to *Polycomb* formation and/or function.

It may also be desirable to include some intron sequences in the promoter constructs since the inclusion of intron sequences in the coding region may result in enhanced expression and specificity. Thus, it may be advantageous to join the DNA sequences to be expressed to a promoter sequence that contains the first intron and exon sequences of a polypeptide which is unique to cells/tissues of a plant critical to seed specific Polycomb formation and/or function.

Additionally, regions of one promoter may be joined to regions from a different promoter in order to obtain the desired promoter activity resulting in a chimeric promoter. Synthetic promoters which regulate gene expression may also be used. The expression system may be further optimized by employing supplemental elements such as transcription terminators and/or enhancer elements.

OTHER REGULATORY ELEMENTS

In addition to a promoter sequence, an expression cassette or construct should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region or polyadenylation signal may be obtained from the same gene as the promoter sequence or may be obtained from different genes. Polyadenylation sequences include, but are not limited to the *Agrobacterium* octopine synthase signal (Gielen et al., EMBO J. (1984) 3:835-846) or the nopaline synthase signal (Depicker et al., Mol. and Appl. Genet. (1982) 1:561-573).

MARKER GENES

Recombinant DNA molecules containing any of the DNA sequences and promoters described herein may additionally contain selection marker genes which encode a selection gene product which confer on a plant cell resistance to a chemical agent or physiological stress, or confers a distinguishable phenotypic characteristic to the cells such that plant cells transformed with the recombinant DNA molecule may be easily selected using a selective agent. One such selection marker gene is neomycin phosphotransferase (NPT II) which confers resistance to kanamycin and the antibiotic G-418. Cells transformed with this selection marker gene may be selected for by assaying for the presence in vitro of phosphorylation of kanamycin using techniques described in the literature or by testing for the presence of the mRNA coding for the NPT II gene by Northern blot analysis in RNA from the tissue of the transformed plant. Polymerase chain reactions are also used to identify the presence of a transgene or expression using reverse transcriptase PCR amplification to monitor expression and PCR on genomic DNA. Other commonly used selection markers include the ampicillin resistance gene, the tetracycline resistance and the

hygromycin resistance gene. Transformed plant cells thus selected can be induced to differentiate into plant structures which will eventually yield whole plants. It is to be understood that a selection marker gene may also be native to a plant.

TRANSFORMATION

A recombinant DNA molecule whether designed to inhibit expression or to provide for expression containing any of the DNA sequences and/or promoters described herein may be integrated into the genome of a plant by first introducing a recombinant DNA molecule into a plant cell by any one of a variety of known methods. Preferably the recombinant DNA molecule(s) are inserted into a suitable vector and the vector is used to introduce the recombinant DNA molecule into a plant cell.

The use of Cauliflower Mosaic Virus (CaMV) (Howell, S.H., et al, 1980, Science, 208:1265) and gemini viruses (Goodman, R.M., 1981, J. Gen Virol. 54:9) as vectors has been suggested but by far the greatest reported successes have been with Agrobacteria sp. (Horsch, R.B., et al, 1985, Science 227:1229-1231).

Methods for the use of Agrobacterium based transformation systems have now been described for many different species. Generally strains of bacteria are used that harbor modified versions of the naturally occurring Ti plasmid such that DNA is transferred to the host plant without the subsequent formation of tumors. These methods involve the insertion within the borders of the Ti plasmid the DNA to be inserted into the plant genome linked to a selection marker gene to facilitate selection of transformed cells. Bacteria and plant tissues are cultured together to allow transfer of foreign DNA into plant cells then transformed plants are regenerated on selection media. Any number of different organs and tissues can serve as targets from Agrobacterium mediated transformation as described specifically for members of the Brassicaceae. These include thin cell layers (Charest, P.J., et al, 1988, Theor. Appl. Genet. 75:438-444), hypocotyls (DeBlock, M., et al, 1989, Plant Physiol. 91:694-701), leaf discs (Feldman, K.A., and Marks, M.D., 1986, Plant Sci. 47:63-69), stems (Fry J., et al, 1987, Plant Cell Repts. 6:321-325), cotyledons (Moloney M. M., et al, 1989, Plant Cell Repts. 8:238-242) and embryoids (Neuhaus, G., et al, 1987, Theor. Appl. Genet. 75:30-36), or even whole plants using in vacuum infiltration and floral dip or floral spraying transformation procedures available in *Arabidopsis* and *Medicago* at present but likely applicable to other plants in the hear future. It is understood, however, that it may be desirable in some crops to choose a different tissue or method of transformation.

Other methods that have been employed for introducing recombinant molecules into plant cells involve mechanical means such as direct DNA uptake, liposomes, electroporation (Guerche, P. et al, 1987, Plant Science 52:111-116) and micro-injection (Neuhaus, G., et al, 1987, Theor. Appl. Genet. 75:30-36). The possibility of using microprojectiles and a gun or other device to force small metal particles coated with DNA

into cells has also received considerable attention (Klein, T.M. et al., 1987, Nature 327:70-73).

It is often desirable to have the DNA sequence in homozygous state which may require more than one transformation event to create a parental line, requiring transformation with a first and second recombinant DNA molecule both of which encode the same gene product. It is further contemplated in some of the embodiments of the process of the invention that a plant cell be transformed with a recombinant DNA molecule containing at least two DNA sequences or be transformed with more than one recombinant DNA molecule. The DNA sequences or recombinant DNA molecules in such embodiments may be physically linked, by being in the same vector, or physically separate on different vectors. A cell may be simultaneously transformed with more than one vector provided that each vector has a unique selection marker gene. Alternatively, a cell may be transformed with more than one vector sequentially allowing an intermediate regeneration step after transformation with the first vector. Further, it may be possible to perform a sexual cross between individual plants or plant lines containing different DNA sequences or recombinant DNA molecules preferably the DNA sequences or the recombinant molecules are linked or located on the same chromosome, and then selecting from the progeny of the cross, plants containing both DNA sequences or recombinant DNA molecules.

Expression of recombinant DNA molecules containing the DNA sequences and promoters described herein in transformed plant cells may be monitored using Northern blot techniques and/or Southern blot techniques or PCR-based methods known to those of skill in the art.

A large number of plants have been shown capable of regeneration from transformed individual cells to obtain transgenic whole plants. For example, regeneration has been shown for dicots as follows: apple, Malus pumila (James et al., Plant Cell Reports (1989) 7:658); blackberry, *Rubus*, Blackberry/raspberry hybrid, *Rubus*, red raspberry, *Rubus* (Graham et al., Plant Cell, Tissue and Organ Culture (1990) 20:35); carrot, *Daucus carota* (Thomas et al., Plant Cell Reports (1989) 8:354; Wurtele and Bulka, Plant Science (1989) 61:253); cauliflower, *Brassica oleracea* (Srivastava et al., Plant Cell Reports (1988) 7:504); celery, *Apium graveolens* (Catlin et al., Plant Cell Reports (1988) 7:100); cucumber, *Cucumis sativus* (Trulson et al., Theor. Appl. Genet. (1986) 73:11); eggplant, *Solanum melonoena* (Guri and Sink, J. Plant Physiol. (1988) 133:52) lettuce, *Lactuca sativa* (Michelmore et al., Plant Cell Reports (1987) 6:439); potato, *Solanum tuberosum* (Sheerman and Bevan, Plant Cell Reports (1988) 7:13); rape, *Brassica napus* (Radke et al., Theor. Appl. Genet. (1988) 75:685; Moloney et al., Plant Cell Reports (1989) 8:238); soybean (wild), *Glycine canescens* (Rech et al., Plant Cell Reports (1989) 8:33);

strawberry, *Fragaria x ananassa* (Nehra et al., Plant Cell Reports (1990) 9:10; tomato, *Lycopersicon esculentum* (McCormick et al., Plant Cell Reports (1986) 5:81); walnut, *Juglans regia* (McGranahan et al., Plant Cell Reports (1990) 8:512); melon, *Cucumis melo* (Fang et al., 86th Annual Meeting of the American Society for Horticultural Science Hort. Science (1989) 24:89); grape, *Vitis vinifera* (Colby et al., Symposium on Plant Gene Transfer, UCLA Symposia on Molecular and Cellular Biology J Cell Biochem Suppl (1989) 13D:255; mango, *Mangifera indica* (Mathews, et al., symposium on Plant Gene Transfer, UCLA Symposia on Molecular and Cellular Biology J Cell Biochem Suppl (1989) 13D:264);

and for the following monocots: rice, *Oryza sativa* (Shimamoto et al., Nature (1989) 338:274); rye, *Secale cereale* (de la Pena et al., Nature (1987) 325:274); maize, (Rhodes et al., Science (1988) 240:204).

In addition, regeneration of whole plants from cells (not necessarily transformed) has been observed in apricot, *Prunus armeniaca* (Pieterse, Plant Cell Tissue and Organ Culture (1989) 19:175); asparagus, *Asparagus officinalis* (Elmer et al., J. Amer. Soc. Hort. Sci. (1989) 114:1019);

Banana, hybrid *Musa* (Escalant and Teisson, Plant Cell Reports (1989) 7:665); bean, *Phaseolus vulgaris* (McClean and Grafton, Plant Science (1989) 60:117); cherry, hybrid Prunus (Ochatt et al., Plant Cell Reports (1988) 7:393); grape, *Vitis vinifera* (Matsuta and Hirabayashi, Plant Cell Reports, (1989) 7:684; mango, *Mangifera indica* (DeWald et al., J Amer Soc Hort Sci (1989) 114:712); melon, *Cucumis melo* (Moreno et al., Plant Sci letters (1985) 34:195); ochra, *Abelmoschus esculentus* (Roy and Mangat, Plant Science (1989) 60:77; Dirks and van Buggenum, Plant Cell Reports (1989) 7:626); onion, hybrid *Allium* (Lu et al., Plant Cell Reports (1989) 7:696); orange, *Citrus sinensis* (Hidaka and Kajikura, Scientia Horiculturae (1988) 34:85); papaya, *Carrica papaya* (Litz and Conover, Plant Sci Letters (1982) 26:153); peach, *Prunus persica* and plum, *Prunus domestica* (Mante et al., Plant Cell Tissue and Organ Culture (989) 19:1); pear, *Pyrus communis* (Chevreau et al., Plant Cell Reports (1988) 7:688; Ochatt and Power, Plant Cell Reports (1989) 7:587); pineapple, *Ananas comosus* (DeWald et al., Plant Cell Reports (1988) 7:535);

watermelon, *Citrullus vulgaris* (Srivastava et al., Plant Cell Reports (1989) 8:300); wheat, *Triticum aestivum* (Redway et al., Plant Cell Reports (1990) 8:714).

The regenerated plants are transferred to standard soil conditions and cultivated in a conventional manner. After the expression or inhibition cassette is stably incorporated into regenerated transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

It may be useful to generate a number of individual transformed plants with any recombinant construct in order to recover plants free from any position effects. It may also be preferable to select plants that contain more than one copy of the introduced recombinant DNA molecule such that high levels of expression of the recombinant molecule are obtained.

As indicated above, it may be desirable to produce plant lines which are homozygous for a particular gene. In some species this is accomplished rather easily by the use of anther culture or isolated microspore culture. This is especially true for the oil seed crop *Brassica napus* (Keller and Armstrong, Z. flanzenzucht 80:100-108, 1978). By using these techniques, it is possible to produce a haploid line that carries the inserted gene and then to double the chromosome number either spontaneously or by the use of colchicine. This gives rise to a plant that is homozygous for the inserted gene, which can be easily assayed for if the inserted gene carries with it a suitable selection marker gene for detection of plants carrying that gene. Alternatively, plants may be self-fertilized, leading to the production of a mixture of seed that consists of, in the simplest case, three types, homozygous (25%), heterozygous (50%) and null (25%) for the inserted gene. Although it is relatively easy to score null plants from those that contain the gene, it is possible in practice to score the homozygous from heterozygous plants by southern blot analysis in which careful attention is paid to the loading of exactly equivalent amounts of DNA from the mixed population, and scoring heterozygotes by the intensity of the signal from a probe specific for the inserted gene. It is advisable to verify the results of the southern blot analysis by allowing each independent transformant to self-fertilize, since additional evidence for homozygosity can be obtained by the simple fact that if the plant was homozygous for the inserted gene, all of the subsequent plants from the selfed seed will contain the gene, while if the plant was heterozygous for the gene, the generation grown from the selfed seed will contain null plants. Therefore, with simple selfing one can easily select homozygous plant lines that can also be confirmed by southern blot analysis.

Creation of homozygous parental lines makes possible the production of hybrid plants and seeds which will contain a modified protein component. Transgenic homozygous parental lines are maintained with each parent containing either the first or second recombinant DNA sequence operably linked to a promoter. Also incorporated in this scheme are the advantages of growing a hybrid crop, including the combining of more valuable traits and hybrid vigor.

The following examples serve to better illustrate the invention described herein and are not intended to limit the invention in any way. All references cited herein are hereby expressly incorporated to this document in their entirety by reference.

EXAMPLE 1
Expression of *MEA* in the Female Gametophyte and in Both Products of Fertilization

Although both the embryo and the endosperm are affected in seeds inheriting a mutant *mea* allele from the female gametophyte, the complex interactions between these tissues do not allow a distinction between primary and secondary effects based on a morphological characterization. To gain insight into the spatial and temporal pattern of *MEA* gene expression, *in situ* hybridization (ISH) was performed using digoxygenin-labeled *MEA* probes. In the ovule, *MEA* mRNA was detected in the 8-nucleated non-cellularized female gametophyte (Fig. 1A). After cellularization, the *MEA* transcript was detected in several cells of the unfertilized embryo sac: the synergids, the egg cell, and the central cell (Fig. 1B, C). In contrast to the synergrids where the signal is present in the cytoplasm, the *MEA* transcript is in close association with the nuclei of the egg cell and the central cell. *MEA* mRNA is not detected in any floral organs, including sepals, petals, stamens, or carpels at any stage of development. No signal could be detected in developing or mature pollen grains. After fertilization, *MEA* mRNA was detected in all cells of the suspensor and the embryo proper (Fig. 1D, E). The transcript persists at a high level to the heart and torpedo stage (Fig. 1F-H), but gradually becomes weaker as embryos reach the cotyledonary stage (Fig. 1I). During the free nuclear phase of endosperm formation, *MEA* mRNA is abundant in dense regions of free nuclei, accumulating at the micropylar and chalazal poles of the embryo sac (Fig. 1D, J). The transcript becomes undetectable as the free nuclei start to cellularize at the periphery of the central cell. Thus, *MEA* mRNA is maternally transcribed in the female gametophyte, and *MEA* mRNA is detectable in both the embryo and the endosperm after fertilization. The high levels of *MEA* mRNA detected in late heart and torpedo stage embryos (Fig. 1H) and in free nuclear endosperm prior to cellularization (Fig. 1J) cannot be accounted for by maternal expression in the egg and central cell only, suggesting that *MEA* is zygotically transcribed.

EXAMPLE 2
The *mea* Maternal Effect is not Caused by a Dosage Effect in the Endosperm

It has been previously shown that *mea* is not a haplo-insufficient locus by adding an extra wild-type *MEA* allele to both embryo and endosperm using a tetraploid pollen donor. Grossnicklaus et al. (1998). The molecular and genetic basis of ovule and megagametophtye development. *Sem. Cell and Dev. Biol.* 9:227-238. However, endosperm derived from these crosses carried an equal number of mutant and wild-type alleles and it is possible that an excess of the wild-type product is required to ensure normal seed development. This hypothesis was tested by analyzing the seed phenotype in duplex

tetraploid plants that carry two mutant *mea-1* and two wild-type *MEA* alleles. The progeny of self-fertilized duplex tetraploids carries between 0 and 6 mutant *mea-1* copies in the endosperm providing the material to differentiate between a dosage effect and maternal inheritance (Figure 3). If an excess of wild-type *MEA* product was required for normal seed development, i.e. four or more wild-type *MEA* copies have to be present in the hexaploid endosperm, it would be expected that 31.5% of the developing seeds to be normal. Alternatively, if the *mea* maternal effect depends on the maternal gametophytic genotype only, i.e. at least one wild-type *MEA* allele has to be inherited from the mother, 78.8% of the developing ovules are expected to form normal seeds. Based on the pooled data of 15 duplex *mea-1* plants where seed abortion was characterized (Table 1), 79.3% (1015/1280 = 0.793) of the developing seeds were found to be normal, strongly suggesting that the ratio of mutant *mea-1* to wild-type *MEA* alleles in the endosperm does not have an effect on seed development, but that seed abortion is solely determined by the maternally inherited allele ($X^2=0.19<\chi^2_{0.05[1]}=3.84$).

Table 1

Observed/Expected	Normal Seeds	Aborted Seeds	X^2
Observed	1015	265	-
Maternal Inheritance Model	1009	271	0.19
Gene Dosage Model	403	877	1356.5

EXAMPLE 3

Transcription of Only Two of the Three *MEA* Copies in the Endosperm After Fertilization

To differentiate between a maternal effect of cytoplasmic or chromosomal nature (imprinting), the expression of the *mea* locus during seed development was characterized. An improved ISH procedure allowed the detection of nuclear dots in the central cell (Fig. 3A). Nuclear dots of intense staining have not previously been reported in plants but they have been observed in mammalian and *Drosophila* nuclei where they were shown to be tightly associated with nascent transcripts of actively expressed genes. Because the nuclear dots are associated with a transcribed genomic locus, they allow an analysis of the transcriptional state of this locus at any given time in development. It was confirmed that the nuclear dots represented nascent transcription sites associated with a genomic locus by

hybridizing *MEA* riboprobes to ovules of a tetraploid plant. As expected, the number of nuclear dots was doubled in the nuclei of a tetraploid plant as compared to a diploid one (Fig. 3B) confirming that the nuclear dots are correlated with the number of *MEA* loci present in these nuclei. The absence of nuclear dots in sections that were incubated with RNAse prior to hybridization indicates that the signal is due to the presence of nascent RNA and not to hybridization to chromosomal DNA (data not shown). Thus, an analysis of nuclear dots allows a determination of the transcriptional state of all *mea* loci present in the central cell nucleus at a specific time in development.

Nascent transcripts could only be visualized in the polar nuclei of the central cell. Nuclear dots were not observed in the smaller nuclei of the egg cell and synergids. To determine the transcriptional state of the *mea* loci in the central cell nucleus before and after fertilization, nuclear dots were analyzed in wild-type diploid plants. Prior to fertilization, a single dot was detected in each of the two haploid polar nuclei present in the central cell (Fig. 3A). Following fusion of the two polar nuclei, two dots were detected in the resulting homo-diploid nucleus (Fig. 3C). At fertilization this homo-diploid nucleus fuses with a sperm nucleus to form the triploid primary endosperm nucleus. In this triploid nucleus only two dots persist after fertilization (results not shown) and following the first mitotic division (Fig. 3D and 3E). The nuclear dots are no longer detectable after a few free nuclear divisions as the nuclei decrease in size. Together, these results show that the *mea* locus is actively transcribed after fertilization during early endosperm development. However, only two nuclear dots are detectable before as well as after fertilization, strongly suggesting that the paternally inherited *MEA* allele remains transcriptionally silent after the fertilization of the central cell.

EXAMPLE 4

Paternally inherited *MEA* alleles are Not Expressed in Either Embryo or Endosperm

To independently confirm that post-fertilization transcription of *MEA* is restricted to the maternally inherited alleles, *MEA* expression by reverse transcription-polymerase chain reaction (RT-PCR) was examined on RNA isolated from developing siliques derived from reciprocal crosses between wild-type and *mea* plants approximately 54 hours after pollination (HAP) when the embryos have reached the mid-globular stage. To obtain homogeneous material resulting from reciprocal crosses, plants were generated that were homozygous for *mea-1*. Although mature desiccated *mea* seeds do not germinate, seedlings can be obtained by culturing embryos *in vitro* or by precocious germination of seeds prior to desiccation. Many of these seedlings show initial developmental aberrations but grow into adult plants that do not show any obvious mutant vegetative or floral

phenotypes. Plants homozygous for *mea-1* are indistinguishable from heterozygotes except that they produce siliques containing 100% aborted seeds as compared to the 50% in a heterozygote. To distinguish between the mutant *mea-1* and the wild-type *MEA* allele, primers were designed that specifically amplify the *mea-1* allele under conditions where the wild-type allele is undetectable (Fig. 4). In reciprocal crosses *mea-1* mRNA can only be detected if it is inherited maternally, but not if inherited from the pollen donor. These results show that paternally inherited *MEA* alleles are not transcribed in either embryo or endosperm early during seed development, since both fertilization products were present in the tissue used for RNA isolation.

EXAMPLE 5
Genomic Imprinting at the *mea* Locus is Not Allele-Specific

The genetic and molecular analysis of *MEA* expression strongly suggests that he *mea* locus is regulated by genomic imprinting. To date, three plant genes have been identified that are likely to be imprinted, all of which are expressed in the endosperm of maize. In each case, only specific alleles are subject to epigenetic regulation by imprinting. In contrast, imprinting in mice is generally locus-specific and all alleles are subject to imprinting. Because our analysis of the transcriptional regulation of *MEA* was only based on a single allele, it was desired to test whether other *MEA* alleles were also regulated by genomic imprinting. To this aim 21 *Arabidopsis* ecotypes were crossed (see Materials and Methods) to homozygous *mea-1/mea-1* female plants to test whether they could support normal seed development. Naturally occurring *MEA* alleles which are not paternally silenced are expected to provide zygotic activity which may be sufficient to rescue the *mea* phenotype. On average 89 seeds were scored per ecotype cross (ranging from 42 to 150 seeds) and no cross produced a significant amount of wild-type seeds that would indicate a lack of paternal silencing. Seventeen of the crosses produced exclusively aborted seeds whereas four produced a few wild-type seeds. The latter occurred at a low frequency (between 1% and 10%) that we attribute to a genetic background effect but not to allele-specific differences in paternal silencing (see below). The production of a few wild-type seeds in these hybrids is likely to be due to instability of paternal silencing during seed development. These findings suggest that none of the naturally occurring *MEA* alleles tested could provide zygotic activity to support normal seed development, indicating that they are all paternally silenced. Thus, unlike at other imprinted loci in plants genomic imprinting of *MEA* is locus- and not allele-specific.

EXAMPLE 6
Ddm1 is a Zygotic Modifier of Genomic Imprinting

Because *MEA* is expressed both maternally and zygotically, it is not clear whether parent-specific expression after fertilization is relevant to the phenotype observed in *mea* mutants. It is possible that *MEA* is only required during a short time before fertilization and that a lack of *MEA* activity in the female gametophyte causes seed abortion later in development. Alternatively, post-fertilization expression of *MEA*, which is under the control of genomic imprinting, may be responsible for the *mea* phenotype. To distinguish between these possibilities, it was attempted to manipulate *MEA* expression in the developing seed. For instance, suppression of the *mea* seed abortion phenotype by post-fertilization expression of *MEA* would suggest that zygotic *MEA* activity is sufficient for normal seed development. Ecotype hybrids gave a first indication that this might be the case since some hybrids produced a small percentage of wild-type seeds.

To further investigate the respective role of maternal and zygotic *MEA* expression, potential modifiers of *mea* were screened among mutants known to affect DNA methylation or gene silencing such as the *decreased in DNA methylation1 (ddm1)* mutant. Recently, *DDM1* which reduces genomic DNA methylation to 30%, has been shown to encode a chromatic remodeling factor of the SW12/SNF2 family. Jeddeloh et al. (1999), Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nature Genetics* 22:94-97. Progeny from crosses involving a *mea-1* plant and a *ddm1-2/DDM1* heterozygote segregated two classes of *mea-1* heterozygous plants that differed in their seed abortion frequency (Table 2). One class showed an abortion frequency (47.5%) close to the one expected for *mea-1/MEA* (50%), the other had a significantly lower abortion rate (39.7%). The two classes segregated in a 1:1 ratio (23:25,X^2=0.083<$\chi^2_{0.05[1]}$=3.84) suggesting that *ddm1* acts as a suppresser of *mea* seed abortion. All plants produced slightly more normal seeds than expected which we attribute to a weak suppression of *mea* seed abortion due to a genetic background effect. The *ddm1-2* allele is in the Columbia (Col) ecotype whereas *mea-1* is in Landsberg *erecta* (Ler). Thus, the plants analyzed were all Col/Ler hybrids. It has been previously noted that Col acts as a weak suppresser of *mea* in out-crosses of *mea-1/MEA* with Col males. It was observed that the mutant maternal *mea-1* allele was transmitted to 3.4% (13/378 = 0.034) of the progeny from these crosses. This is in good agreement with the observed increase of wild-type seeds by 3.6% from the expected 50% to 53.6% (5025/9369 = 0.536) among all plants analyzed (Table 2).

Plants heterozygous for the *ddm1-2* allele did not only show a decrease in the frequency of aborted seeds but also produced seeds that were considerably larger than their wild-type siblings (Figure 6). They were still green when the wild-type seeds had lost their chlorophyll pigmentation suggesting a delay in seed development. These enlarged seeds

were observed at a frequency of 5.7% (Table 2) which suggests that they were homozygous for *ddm1-2*. We expect 1/8th of the seeds that inherit a maternal *mea-1*^{m} allele to also be homozygous for *ddm1-2* and to carry a wild-type paternal *MEA*^{p} allele (0.5 * ¼*1/2=0.0625; without correction for the Col/*Ler* background effect). Given that 3.6% of the seeds which inherit a mutant maternal *mea-1* ^{m} allele survive in this genetic background, a frequency of 5.7% is in very good agreement with the expected 5.8% [(0.5 - 0.036) * 1/8 = 0.058; with correction for the genetic background effect]. The observed segregation ratio strongly suggests that seeds inheriting a maternal *mea-1* allele which usually abort, are rescued to maturity if they are also homozygous for *ddm1-2*. That homozygosity of *ddm1* in the developing seed is required for suppression of the *mea* phenotype was confirmed by out-crossing these plants to *mea-1/mea-1* homozygotes. If *ddm1* acted in the male gametophyte and prevented the silencing of the paternal *MEA*^{p} allele, 25% of the pollen from a *mea-1/MEA; ddm1-2/* DDM1 plant should carry a non-silenced wild-type *MEA* allele(pollen genotype: *MEA*^{p};*ddm1-2*) expected to rescue 25% of the seeds. Among 259 seeds scored, no rescued seeds were observed in these crosses. Furthermore, the predicted genotype (*mea-1*^{m}*/MEA*^{p} ; *ddm1-2/ddm1-2*) was confirmed of the enlarged seeds by scoring kanamycin resistance associated with the *mea-1* allele and by confirming homozygosity for *ddm1* by Southern analysis or scoring a CAPS marker linked to the *ddm1-2 allele* (data not shown). These genetic analyses strongly suggest that *ddm1* acts as a zygotic modifier of *mea*, i.e. seeds carrying a mutant maternal *mea-1* allele survive to maturity if they are also homozygous for *ddm1*. In seeds of this genotype, *MEA* activity is likely to be provided by the paternally inherited *MEA* allele that gets re-activated during seed development due to a lack of *DDM1* activity. This is supported by the fact that all 15 plants derived from enlarged seeds that we tested were heterozygous for *mea-1* and, thus, had inherited a wild-type *MEA* allele from the father. If the effect of *ddm1* were bypassing *MEA* rather than reactivating the paternal copy, it would be expected that half the plants to inherit a mutant *mea-1* allele from the father and twice more *mea* seeds should survive than observed. These findings strongly suggest that zygotic *MEA* activity provided from a re-activated paternal allele is sufficient to support seed development. Thus, post-fertilization expression of *MEA*, which is subject to genomic imprinting, is responsible for the *mea* phenotype.

Table 2

Seed Abortion in mea Heterozygotes Segregating for ddm1-2

Genotype	Aborted Seeds (%)	Normal Seeds (%)	Enlarged Seeds (%)	N[scored]
mea-1/MEA;DDM1/DDM1	47.5	52.5	0	4361
mea-1/MEA;ddm1-2/DDM1	39.7	54.6	5.7	5008

EXAMPLE 7

mea Seeds Rescued by *ddm1* Show Overgrowth of the Embryo and Persistent Endosperm

At maturity, the viability of large seeds lacking *DDM1* activity is comparable to the wild type but their germination frequency is reduced by 18% after prolonged storage (124/157 = 0.79 for enlarged seeds vs. 126/130 = 0.97 for wild-type siblings). Except for their enlarged size many embryos in these seeds are normal although some aberrations were observed. At maturity, three different classes of enlarged seeds can be distinguished (Figure 7A to 7D). In the predominant class (50/111 = 0.45), most of the seed volume is occupied by a large, well organized embryo that is normally curved, with both cotyledons parallel to each other (Figure 7B). In these embryos aberrant cell proliferation in the hypocotyl and the cotyledons causes enlarged tissue sectors (Figure 7E) with small irregular cells present in both epidermal and subepidermal layers (Figure 7G). These cells appear to be poorly differentiated, but their presence does not compromise the overall morphology of the embryo. In contrast to wild-type seeds, a large portion of partially cellularized endosperm persists between the hypocotyl and the cotyledons (Figure 7H). due to their large size (diameter 1.6 x larger than the wild type), these embryos sometimes break the seed coat in the cotyledonary region. The second class of rescued seeds (46/111 = 0.41) are characterized by the presence of massive amounts of partially cellularized endosperm (Figure 7D) persisting in both the micropylar and chalazal chambers. The size of the tissue and its degree of cellularization appear to be variable. Embryos have a normal morphology with enlarged tissue sectors in the hypocotyl or the cotyledons; however, growth of the bent cotyledons appears to be limited and the embryos only reach the "walking stick" stage (Bowman, 1994) of development. The region which is not occupied

by the cotyledons is filled with persistent endosperm. Seeds of the third class (15/111 = 0.14) contain mature embryos that have aberrant morphology. In some seeds (6%) the cotyledons are perpendicular to the apical-basal axis (T-shaped embryos; Figure 7C) whereas in other seeds (8%), the embryos are twisted in a spiral-like fashion that affects the whole length of the hypocotyl (data not shown). Due to these morphological aberrations, seeds are often swollen and round, with a large portion of partially cellularized endosperm surrounding the embryo. Taken together, these results show that most rescued seeds have enlarged embryos that often complete morphogenesis. Their defects suggest that they initially show the *mea* phenotype, which is characterized by delayed morphogenesis and abnormal cell proliferation as compared to wild-type siblings. However, and in contract to *mea* seeds, enlarged embryos are able to resume embryogenesis and often complete their development. Differences in germination frequency between wild-type and enlarged seeds (18%) suggest that it is only the small proportion of enlarged seeds showing dramatic morphological defects (about 14%) that cannot withstand full desiccation to give rise to viable seedlings.

Materials and Methods for Examples 1-7

Plant Materials and Growth Conditions

The *mea-1* mutant used in this study was described by Grossniklaus et al. (1998). The duplex tetraploid plants carrying two copies of *mea-1* were obtained in the progeny of a self-pollinated simplex *mea-1* plant described previously. The *ddm1-2* mutant was kindly provided by Eric Richards. The ecotypes used in this study were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University (stock numbers in parenthesis). Aa-0, Aua/Rhön-Germany (CS0900); C24 (CS0906); Da(1)-12, Darmstadt-CSFR (CS0917); En-D, Donetsk-Ukraine (CS0920); Litva, Lithuania (CS0925); Ca-0, Camberg/Taunus-Germany (CS1060); Ct-1, Catania-Italy (CS1094); Db-0, Dombachtal/Ts-Germany (CS1100); Di-0, Dijon-France (CS1006); Ga-0, Gabelstein-Germany (CS1180); J1-1, Vranov u Brno-CSFR (CS1258); Kn-0, Kaunas-Russia (CS1286); Lc-1, Loch Ness-Scotland (CS1306); Mt-0, Martuba/Cyrenaika-Libya (CS1380); Nd-0, Niederzenz-Germany (CS1390); Tsu-0, Tsu-Japan (CS1564); Wil-1, Wilna-Lituania (CS1594); Oy-1, Oyetese-Norway (CS1643); Wei-0, Weiningen-Switzerland (CS3110); Shahdara, Pamiro/Alay-Tadjikistan (CS6180); Berkeley, Berkeley-USA (CS8068). Growth conditions were describe in Moore et al. (1997). Genetic characterization of hadad, a mutant disrupting megagametogenesis in Arabidopsis thaliana. *Cold Spring Harbor Symp. Quant. Biol* 62:35-47.

In situ hybridization

In situ hybridization was performed as described with modifications needed for improved resolution in developing female gametophytes of *Arabidopsis*. For synthesis of sense and anti-sense 11-digoxigenin-UTP labeled probes, a plasmid pGEM7Z (Promega) containing a 290 bp fragment (5' end of the MEA cDNA) was linearized with restriction enzymes cutting in the polylinker and 1 μg was used as a template for probe synthesis. Whole inflorescences, dissected carpels, and developing siliques were fixed in 4% paraformaldehyde and embedded in Paraplast. Sections at 12 to 15 μm thickness were cut using a Reichert microtome and attached to ProbeOnPlus slides (Fisher Biotech). After dewaxing and hydration, slides were digested with 0.125 mg/ml Pronase E (SIGMA) for 28 minutes at 37°C. Following a 2 minutes postfixation in 4% paraformaldehyde, slides were dehydrated and immediately processed for hybridization. RNA probes were hydrolyzed as described, and 3% to 6% of each labeling reaction (400 to 800 ng of RNA) were mixed with 40 μl 50% formamide, added to 200 μl of hybridization at 55°C, the slides were washed twice with gentle agitation in 0.2X SSC for 1 hour at 55°C, followed by two rinses at room temperature (25°C) in NTE (0.5 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA), and treated with 20 mg/ml of Rnase in the same buffer at 37°C for 30 minutes. They were subsequently rinsed in fresh NTE and washed again in 0.2X SSC for 1 hour at 55°C. For immunological detection, slides were incubated twice (45 and 30 minutes) with gentle agitation in 0.5% blocking agent (Bohehringer-Mannheim) in TBS (100 mM Tris-HCl pH 7.5, 150 mM NaCl), followed by 45 minutes in 1% BSA, 0.3% Triton X-100 in TBS. This was followed by a 2 hour incubation in anti-digoxygenin conjugated antibody diluted 1:1250 in 1% BSA, 0.3% Triton X-100 in TBS, and four washes of 20 minutes in the same buffer. The slides were washed twice for 15 minutes in buffer C (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl), and incubated for 2 to 5 days in 0.34 mg/ml nitroblue tetrazolium salt (NBT) and 0.175 mg/ml 5-bromo-4-chloro-3-indyl phosphate toluidinium salt (BCIP) in buffer C containing 7.6 mM levamisole (SIGMA). After stopping the reaction with 10 mM Tris-HCl, 1 mM EDTA, slides were dehydrated through an ethanol series, mounted in Cytoseal (Stephens Scientific), and analyzed with a Leica DMRB microscope under brightfield or Nomarski optics.

Reverse Transcription PCR Analysis

For RNA preparation young siliques were harvested between 52 and 56 HAP in liquid nitrogen. For accurate comparisons control siliques of self-fertilized L*er* wild-type plants and *mea-1* homozygous plants (*mea-1/mea-1*) were emasculated and hand pollinated in the same way as the reciprocal crosses between L*er* and *mea-1/mea-1*. RNA was prepared using the Trizol LS reagent (GIBCO-BRL). For RT-PCR approximately 5 μg of total RNA were treated with 5 units of RNAse-free DNAse (Boehringer-Mannheim) in 1X PCR buffer (GIBCO-BRL) containing 2.5 mM $MgCl_2$ at 37°C for 30 minutes. After heat

inactivation at 80°C for 5 minutes, samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol. The RNA was reverse transcribed using 5 pmoles of random hexamers (Pharmacia Biotech) in a 12 µl reaction containing 1X PCR buffer (GIBCO-BRL), 2.1 mM $MgCl_2$, 0.5 mM of each deoxynucleotide triphosphate (dNTP), 10 mM dithiothreitol, and 120 units Superscript II reverse transcriptase (GIBCO-BRL) by incubating at 25°C for 10 minutes followed by 42°C for 55 minutes and heat inactivation at 70°C for 20 minutes. 1/4 of the cDNA samples was used for PCR amplification of MEA, 1/12 of the samples was used to amplify *ACT11*, and 0.5 ng of genomic *Ler* DNA for the controls. PCR was performed in 1X PCR buffer (Perkin Elmer) containing 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 unit of Taq DNA polymerase (Perkin-Elmer/Cetus), TaqStart antibody (Clontech) in a molar ratio of 28:1 relative to Taq DNA polymerase, and 20 pmoles of each gene specific primer for 30 cycles at an annealing temperature of 58°C. The primers used for amplification of *ACT11* were as described. For *MEA*, the primers which specifically amplify the *mea-1* allele under these conditions, were: meaS12 (5'-CTCATGATGAAGCTAATGAGC-3')(SEQ ID NO:2) and meaAS11 (5'GCATGTTCTGGTCCATAGC-3')(SEQ ID NO:3).

Histological Analysis

For whole mount specimens, siliques at various stages of development were dissected with hypodermic needles (Beckton-Dickinson, 1cc insulin syringes). Individual seeds and embryos were isolated and mounted for photography under a Leica dissecting stereoscope. For sectioned material, individual seeds were fixed in 3% glutaraldehyde for 2 hours, rinsed in 50 mM cacodylate buffer (EMS) and postfixed in 2% osmium tetroxyde (in same buffer). After dehydration in an acetone series, specimens were embedded in Spurr's resin as described. Specimens were sectioned on an UltracutE ultramicrotome (Reichert-Jung) and observed on a Leica DMR microscope under brightfield or Nomarski optics.

As can be seen from the foregoing the invention accomplishes at least all of its objectives.

REFERENCES

Asker, S., and L. Jerling, 1992.. *Apomixis in Plants*. CRC Press, Boca Ratón, FL.

Bowman, J. 1994. *Arabidopsis, an Atlas of Morphology and Development* Springer Verlag, New York, NY.

Burnham, C.R. 1962. *Discussions in Cytogenetics* Burgess Publishing Company, Minneapolis, MN.

Caspary, T., M.A. Cleary, C.C. Baker, X.-J. Guan, and S.M. Tilghman. 1998. Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. *Mol. Cell. Biol.* **18:** 3466-3474.

Castle, L.A., D. Errampalli, T.L. Atherton, L.H. Franzmann, E.S. Yoon, and D.W. Meinke. 1993. Genetic and molecular characterization of embryonic mutants identified following seed transformation in *Arabidopsis*. *Mol. Gen. Genet.* **5:** 363-369.

Chaudhuri, S., and J. Messing. 1994. Allele-specific parental imprinting of *dzr-1*, a post-transcriptional regulator of zein accumulation. *Proc. Natl. Acad. Sci. U. S. A.* **91:** 4867-4871.

Chaudhury, A.M., L. Ming, C. Miller, S. Craig, E.S. Dennis, and W.J. Peacock. 1997. Fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 4223-4228.

Coen, E.S., J.M. Romero, S. Doyle, R. Elliott, G. Murphy, and R. Carpenter. 1990. *floricaula*: a homeotic gene required for flower development in *Antirrhinum majus*. *Cell* **63:** 1311-1322.

Colombo, L., J. Franken, A.R. Van der Krol, P.E. Wittich, H.J. Dons, and G.C. Angenent. 1997. Downregulation of ovule specific MADS box genes from petunia results in maternally controlled defects in seed development. *Plant Cell* **9:** 703-715.

Grossniklaus, U., and K. Schneitz. 1998. The molecular and genetic basis of ovule and megagametophyte development. *Sem. Cell and Dev. Biol.* **9:** 227-238.

Grossniklaus, U., and J-P. Vielle-Calzada. 1998. Response: parental conflict and infanticide during embryogenesis. *Trends in Pl. Sci.* **3:** 328.

Grossniklaus, U., J-P. Vielle-Calzada, M.A. Hoeppner, and W.B. Gagliano. 1998. Maternal control of embryogenesis by *MEDEA*, a *Polycomb* group gene in *Arabidopsis*. *Science* **280:** 446-450.

Haig, D., and C. Graham. 1991. Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell* **54:** 1045-1046.

Haig, D., and M. Westoby. 1989. Parent specific gene expression and the triploid endosperm. *Am. Nat.* **134:** 147-155.

Haig, D., and M. Westoby. 1991. Genomic imprinting in endopserm: its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Phil. Trans. R. Soc. Lond.* **333**: 1-13.

Hong, S.K., H. Kitano, H. Satoh, and Y. Nagato. 1996. How is embryo size genetically regulated in rice? *Development* **7**: 2051-2058.

Imbalzano, A.N., G.R. Schnitzler, and R.E. Kingston. 1994. Facilitated binding of TATA-binding protein to nucleosomal DNA. *Nature* **370**: 481-485.

John, R.M., and M.A. Surani. 1996. Imprinted genes and regulation of gene expression by epigenetic inheritance. *Curr. Opin. Cell. Biol.* **3**: 348-353.

Jackson, D.P. 1991. *In situ* hybridization in plants. In *Molecular Plant Pathology: A Practical Approach* (ed. D.J. Bowles, S.J. Gurr, and M. McPhereson) pp 163-181. Oxford University Press, Oxford, UK.

Jaenisch, R. 1997. DNA methylation and imprinting: why bother? *Trends Genet.* **13**: 323-329.

Jeddeloh, J.A., J. Bender, and E.J. Richards. 1998. The DNA methylation locus *DDM1* is required for maintenance of gene silencing in *Arabidopsis*. *Genes & Dev.* **12**: 1714-1725.

Jeddeloh, J.A., T.L. Stokes, and E.J. Richards. 1999. Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nature Genetics* **22**: 94-97.

Jones, B.K, J. Levorse, and S.M. Tilghman. 1998. *Igf2* imprinting does not require its own DNA methylation or *H19* RNA. *Genes & Dev.* **12**: 2200-2207.

Kakutani, T., J.A. Jeddeloh, S.K. Flowers, K. Munakata, and E.J. Richards. 1996. Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. *Proc. Natl. Acad. Sci. U.S.A.* **29**: 12406-12411.

Kakutani, T. 1997. Genetic characterization of late-flowering traits induced by DNA hypomethylation mutation in *Arabidopsis thaliana*. *The Plant Journal* **12**: 1447-1151.

Kakutani, T., E. Munakata, E.J. Richards, and H. Hirochika. 1999. Meiotically and mitotically stable inheritance of DNA hypomethylation induced by *ddm1* mutation of *Arabidopsis thaliana*. *Genetics* **151**: 831-838.

Kennison, J.A. 1995. The *Polycomb* and *trithorax* group proteins of *Drosophila*: trans-regulators of homeotic gene function. *Annu. Rev. Genet.* **29**: 289-303.

Kermicle, J. 1970. Dependence of the R-motted aleurone phenotype in maize on the mode of sexual transmission. *Genetics* **66**: 69-85.

Kermicle, J., and M. Alleman. 1990. Gametic imprinting in maize in relation to the angiosperm life cycle. *Development (Suppl.)* **1**: 9-14.

Kiyosue, T., N. Ohad, R. Yadegari, M. Hannon, J. Dinneny, D. Wells, A. Katz, L. Margossian, J.J. Harada, R.B Goldberg, and R.L. Fischer. 1999. Control of fertilization independent endosperm development by the MEDEA polycomb gene in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* **96:** 4186-4191.

Koltunow, A.M. 1993. Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. *Plant Cell* **5:** 1425-1437.

Korf, I., Y. Fan, and S. Strome. 1998. The *Polycomb* group in *Caenorhabditis elegans* and maternal control of germline development. *Development* **125:** 2469-2478.

Lawrence, J.B., R.H. Singer, and L.M. Marselle. 1989. Highly localized tracks of specific transcripts within interphase nuclei visualized by *in situ* hybridization. *Cell* **57:** 493-502.

Lin, B-Y. 1984. Ploidy barrier to endosperm development in maize. *Genetics* **107:** 103-115.

Lund, G., J. Messing, and A. Viotti. 1995. Endosperm-specific demethylation and activation of specific alleles of a-tubulin genes of *Zea mays* L. *Mol. Gen. Genet.* **246:** 716-722.

Martienssen, R.A. 1996. Epigenetic phenomena: paramutation and gene silencing in plants. *Curr. Biol.* **6:** 810-813.

Martienssen, R.A. 1998. Chromosomal imprinting in plants. *Curr. Opin. Gen. & Dev.* **8:** 240-244.

Messing J, and U. Grossniklaus. 1999. Genomic imprinting in plants. In *Results and Problems in Cell Differentiation* (ed. R. Ohlsson), pp 23-40. Springer Verlag, Berlin-Heidelberg, Germany.

Ming, L., P. Bilodeau, A. Koltunow, E.S. Dennis, W.J. Peacock, and A.M. Chaudhury. 1999. Genes controlling fertilization-independent seed development in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U.S.A.* **96:** 296-301.

Moore, J.M., J-P. Vielle Calzada, W. Gagliano, and U. Grossniklaus. 1997. Genetic characterization of *hadad*, a mutant disrupting megagametogenesis in *Arabidopsis thaliana*. *Cold Spring Harbor Symp. Quant. Biol* **62:** 35-47.

Neumann, B., and D.P. Barlow. 1996. Multiple roles for DNA methylation in gametic imprinting. *Curr. Opin. Genet. Dev.* **6:** 159-163.

Moore, T., and D. Haig. 1991. Genomic imprinting in mammalian development: a parental though-of-war. *Trends Genet.* **7:** 45-49.

Ohad, N., L. Margossian, Y-C. Hsu, C. Williams, P. Repetti, and R.L. Fischer. 1996. A mutation that allows endosperm development without fertilization. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 5319-5324.

Ohad, N., R. Yadegari, L. Margossian, M. Hannon, D. Michaeli, J.J. Harada, R.B. Goldberg, and R.L. Fischer. 1999. Mutations in FIE, a WD polycomb group gene, allow endosperm development without fertilization. *Plant Cell* **11**: 407-416.

Orlando, V., and R. Paro. 1995. Chromatin multiprotein complexes involved in the maintenance of transcription patterns. *Curr. Opin. Genet. Dev.* **5**: 174-179.

Pelletier, G. 1998. Use of haplo-diploidization for plant breeding. In *Androgenesis and Haploid Plants* (ed. Y. Chupeau, M. Caboche, and Y. Henry), pp104, Springer Verlag, Heidelberg, Germany.

Pirrotta, V. 1997. PcG complexes and chromatin silencing. *Curr. Opin. Genet. Dev* **7**: 249-258.

Ray, A. 1998. New paradigms in plant embryogenesis: maternal control comes in different flavors. *Trends Pl. Sci.* **3**: 325-327.

Ray, S., T. Golden, and A. Ray. 1996. Maternal effects of the *short integument* mutation on embryo development in *Arabidopsis. Dev. Biol.* **180**: 365-369 .

Richards, E.J. 1997. DNA methylation and plant development. *Trends Genet.* **13**: 319-323.

Schnitzler, G., S. Sif, and R.E. Kingston. 1998. Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state. *Cell* **94**: 17-27.

Scott, R., M. Spielman, J. Bailey, and H.G. Dickinson. 1998a. Parent-of-origin effects on seed development in *Arabidopsis thaliana. Development* **125**: 3329-3341.

Scott, R., R. Vinkenoog, M. Spielman, and H.G. Dickinson. 1998b. Medea: murder or mistrial? *Trends Pl. Sci.* **3**: 460-461.

Shermoen, A.W., and P.H. O'Farrell. 1991. Progression of the cell cycle through mitosis leads to abortion of nascent transcripts. *Cell* **67**: 303-310.

Tilghman, S.M. 1999. The sins of the fathers and mothers: genomic imprinting in mammalian development. *Cell* **96**: 185-193.

Reik, W., and E.R. Maher. 1997. Imprinting in clusters: lessons from Beckwith-Wiedemann syndrome. *Trends Genet.* **13**: 330-335.

Utley, R.T., J. Cote, T. Owen-Hughes, and J.L. Workman, J.L. 1997. SWI/SNF stimulates the formation of disparate activator-nucleosome complexes but is partially redundant with cooperative binding. *J. Biol. Chem.* **272**: 12642-12649.

van der Veen, J.H., and H. Blankenstijn-de Vries. 1973. Double reduction in tetraploid *Arabidopsis thaliana*, studied by means of a chlorophyll mutant with a distinct simplex phenotype. *Arabidopsis Inf. Serv.* **10**: 11-12.

Vielle-Calzada, J-P., C.F. Crane, and D.M. Stelly. 1996. Apomixis: the asexual revolution. *Science* **274**: 1322-1323.

Vongs, A., T. Kakutani, R.A. Martienssen, and E.J. Richards, E.J. 1993. *Arabidopsis thaliana* DNA methylation mutants. *Science* **260**: 1926-1928.

Walbot, V. 1996 Sources and consequences of phenotypic and genotypic plasticity in flowering plants. *Trends Pl. Sci.* **1**: 27-32.

We claim:

1. A method of increasing seed size in plants comprising: regulating seed specific Polycomb group gene expression so that gene expression is inhibited or otherwise inactivated during early seed development and is present or activated during late seed development.

2. The method of claim 1 wherein said seed specific Polycomb group gene encodes a regulatory protein characterized by the following: (a) is a Polycomb group member; (b) comprises a SET domain or a CXC domain or both; (c) regulates cell proliferation; and said gene having a nucleotide sequence capable of hybridizing under conditions of high stringency to SEQ ID NO:1.

3. The method of claim 1 wherein said inhibition of Polycomb group gene expression includes a technique selected from the group consisting of: sense co-suppression, antisense co-suppression and double stranded RNA interference.

4. The method of claim 1 wherein said method includes a technique selected from the group consisting of: insertional mutation and homologous recombination.

5. The method of claim 1 wherein said method includes a technique comprising: a second site modifier gene.

6. The method of claim 5 wherein said modifier gene is a gene with a mutation that removes the imprint at the paternally inherited seed specific Polycomb group allele.

7. The method of claim 6 wherein said modifier gene is one with the mutation DDM1.

8. A prokaryotic or eucaryotic host cell transformed or transfected with an expression construct according to claim 1.

9. The cell of claim 8 wherein said cell is a plant cell.

10. A method of producing a plant with larger than average seeds comprising: inhibiting expression of a seed specific Polycomb group gene during early seed

development; and thereafter, resuming expression of said seed during late seed development.

11. The method of claim 10 wherein said early seed development is from fertilization to late heart or torpedo stage.

12. The method of claim 10 wherein said late seed development includes any time after late heart or torpedo stage.

13. A seed produced by the method of claim 1.

14. A plant capable of producing larger than average seeds wherein said plant or an ancestor thereof was produced by the method of claim 1.

15. A method of regulating seed development in plants comprising: introducing to a plant cell a genetic construct said construct being one with an expression or inhibition construct and which will express or inhibit a seed specific Polycomb gene.

16. The method of claim 15 wherein said construct is an expression construct.

17. The method of claim 16 wherein said construct is an inhibition construct.

18. A method for removing the genetic imprint at the *mea* locus and for providing for increased seed size in plants comprising: introducing to a plant cell carrying a *mea* mutant allele a modifier expression construct comprising a nucleotide sequence capable of rescuing the mea mutant phenotype.

19. The method of claim 18 wherein said modifier expression construct comprises a nucleotide sequence which encodes the DDM1 phenotype.

20. The method of claim 18 wherein the plant cell is *Arabidopsis* or *Brassica napus*.

21. The enlarged seed of claim 13 whereby the seed is about 1.6 times larger than wild-type seed.

22. A method of regulating the size of seeds in plants comprising: introducing to a plant cell a genetic construct, said construct being one with an expression or inhibition construct and which will express or inhibit a seed specific DDM1 gene.

23. The method of claim 22 wherein said construct is an expression construct.

24. The method of claim 22 wherein said regulation involves increasing embryo content of a seed.

25. The method of claim 22 wherein said regulation involves decreasing endosperm content of a seed.

26. The method of claim 22 wherein said construct is an inhibition construct.

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```
aggcgagtgg tta atg gag aag gaa aac cat gag gac gat ggt gag ggt    49
               Met Glu Lys Glu Asn His Glu Asp Asp Gly Glu Gly
                 1               5                  10

ttg cca ccc gaa cta aat cag ata aaa gag caa atc gaa aag gag aga    97
Leu Pro Pro Glu Leu Asn Gln Ile Lys Glu Gln Ile Glu Lys Glu Arg
         15                  20                  25

ttt ctg cat atc aag aga aaa ttc gag ctg aga tac att cca agt gtg   145
Phe Leu His Ile Lys Arg Lys Phe Glu Leu Arg Tyr Ile Pro Ser Val
     30                  35                  40

gct act cat gct tca cac cat caa tcg ttt gac tta aac cag ccc gct   193
Ala Thr His Ala Ser His His Gln Ser Phe Asp Leu Asn Gln Pro Ala
 45                  50                  55                  60

gca gag gat gat aat gga gga gac aac aaa tca ctt ttg tcg aga atg   241
Ala Glu Asp Asp Asn Gly Gly Asp Asn Lys Ser Leu Leu Ser Arg Met
                 65                  70                  75

caa aac cca ctt cgt cat ttc agt gcc tca tct gat tat aat tct tac   289
Gln Asn Pro Leu Arg His Phe Ser Ala Ser Ser Asp Tyr Asn Ser Tyr
             80                  85                  90

gaa gat caa ggt tat gtt ctt gat gag gat caa gat tat gct ctt gaa   337
Glu Asp Gln Gly Tyr Val Leu Asp Glu Asp Gln Asp Tyr Ala Leu Glu
         95                  100                 105

gaa gat gta cca tta ttt ctt gat gaa gat gta cca tta tta cca agt   385
Glu Asp Val Pro Leu Phe Leu Asp Glu Asp Val Pro Leu Leu Pro Ser
    110                 115                 120

gtc aag ctt cca att gtt gag aag cta cca cga tcc att aca tgg gtc   433
Val Lys Leu Pro Ile Val Glu Lys Leu Pro Arg Ser Ile Thr Trp Val
125                 130                 135                 140

ttc acc aaa agt agc cag ctg atg gct gaa agt gat tct gtg att ggt   481
Phe Thr Lys Ser Ser Gln Leu Met Ala Glu Ser Asp Ser Val Ile Gly
                145                 150                 155

aag aga caa atc tat tat ttg aat ggt gag gca cta gaa ttg agc agt   529
Lys Arg Gln Ile Tyr Tyr Leu Asn Gly Glu Ala Leu Glu Leu Ser Ser
            160                 165                 170

gaa gaa gat gag gaa gat gaa gaa gaa gat gag gaa gaa atc aag aaa   577
Glu Glu Asp Glu Glu Asp Glu Glu Glu Asp Glu Glu Glu Ile Lys Lys
        175                 180                 185

gaa aaa tgc gaa ttt tct gaa gat gta gac cga ttt ata tgg acg gtt   625
Glu Lys Cys Glu Phe Ser Glu Asp Val Asp Arg Phe Ile Trp Thr Val
    190                 195                 200

ggg cag gac tat ggt ttg gat gat ctg gtc gtg cgg cgt gct ctc gcc   673
Gly Gln Asp Tyr Gly Leu Asp Asp Leu Val Val Arg Arg Ala Leu Ala
205                 210                 215                 220
```

Fig. 1a

```
aag tac ctc gaa gtg gat gtt tcg gac ata ttg gaa aga tac aat gaa   721
Lys Tyr Leu Glu Val Asp Val Ser Asp Ile Leu Glu Arg Tyr Asn Glu
                225                 230                 235

ctc aag ctt aag aat gat gga act gct ggt gag gct tct gat ttg aca   769
Leu Lys Leu Lys Asn Asp Gly Thr Ala Gly Glu Ala Ser Asp Leu Thr
            240                 245                 250

tcc aag aca ata act act gct ttc cag gat ttt gct gat aga cgt cat   817
Ser Lys Thr Ile Thr Thr Ala Phe Gln Asp Phe Ala Asp Arg Arg His
        255                 260                 265

tgc cgt cgt tgc atg ata ttc gat tgt cat atg cat gag aag tat gag   865
Cys Arg Arg Cys Met Ile Phe Asp Cys His Met His Glu Lys Tyr Glu
    270                 275                 280

ccc gag tct aga tcc agc gaa gac aaa tct agt ttg ttt gag gat gaa   913
Pro Glu Ser Arg Ser Ser Glu Asp Lys Ser Ser Leu Phe Glu Asp Glu
285                 290                 295                 300

gat aga caa cca tgc agt gag cat tgt tac ctc aag gtg agg agt gtg   961
Asp Arg Gln Pro Cys Ser Glu His Cys Tyr Leu Lys Val Arg Ser Val
                305                 310                 315

aca gaa gct gat cat gtg atg gat aat gat aac tct ata tca aac aag   1009
Thr Glu Ala Asp His Val Met Asp Asn Asp Asn Ser Ile Ser Asn Lys
            320                 325                 330

att gtg gtc tca gat cca aac aac act atg tgg acg cct gta gag aag   1057
Ile Val Val Ser Asp Pro Asn Asn Thr Met Trp Thr Pro Val Glu Lys
        335                 340                 345

gat ctt tac ttg aaa gga att gag ata ttt ggg aga aac agt tgt gat   1105
Asp Leu Tyr Leu Lys Gly Ile Glu Ile Phe Gly Arg Asn Ser Cys Asp
    350                 355                 360

gtt gca tta aac ata ctt cgg ggg ctt aag acg tgc cta gag att tac   1153
Val Ala Leu Asn Ile Leu Arg Gly Leu Lys Thr Cys Leu Glu Ile Tyr
365                 370                 375                 380

aat tac atg cgc gaa caa gat caa tgt act atg tca tta gac ctt aac   1201
Asn Tyr Met Arg Glu Gln Asp Gln Cys Thr Met Ser Leu Asp Leu Asn
                385                 390                 395

aaa act aca caa aga cac aat cag gtt acc aaa aaa gta tct cga aaa   1249
Lys Thr Thr Gln Arg His Asn Gln Val Thr Lys Lys Val Ser Arg Lys
            400                 405                 410

agt agt agg tcg gtc cgc aaa aaa tcg aga ctc cga aaa tat gct cgt   1297
Ser Ser Arg Ser Val Arg Lys Lys Ser Arg Leu Arg Lys Tyr Ala Arg
        415                 420                 425

tat ccg cct gct tta aag aaa aca act agt gga gaa gct aag ttt tat   1345
Tyr Pro Pro Ala Leu Lys Lys Thr Thr Ser Gly Glu Ala Lys Phe Tyr
    430                 435                 440

aag cac tac aca cca tgc act tgc aag tca aaa tgt gga cag caa tgc   1393
Lys His Tyr Thr Pro Cys Thr Cys Lys Ser Lys Cys Gly Gln Gln Cys
```

Fig. 1b

```
445                 450                 455                 460

cct tgt tta act cac gaa aat tgc tgc gag aaa tat tgc ggg tgc tca   1441
Pro Cys Leu Thr His Glu Asn Cys Cys Glu Lys Tyr Cys Gly Cys Ser
                465                 470                 475

aag gat tgc aac aat cgc ttt gga gga tgt aat tgt gca att ggc caa   1489
Lys Asp Cys Asn Asn Arg Phe Gly Gly Cys Asn Cys Ala Ile Gly Gln
            480                 485                 490

tgc aca aat cga caa tgt cct tgt ttt gct gct aat cgt gaa tgc gat   1537
Cys Thr Asn Arg Gln Cys Pro Cys Phe Ala Ala Asn Arg Glu Cys Asp
        495                 500                 505

cca gat ctt tgt cgg agt tgt cct ctt agc tgt gga gat ggc act ctt   1585
Pro Asp Leu Cys Arg Ser Cys Pro Leu Ser Cys Gly Asp Gly Thr Leu
    510                 515                 520

ggt gag aca cca gtg caa atc caa tgc aag aac atg caa ttc ctc ctt   1633
Gly Glu Thr Pro Val Gln Ile Gln Cys Lys Asn Met Gln Phe Leu Leu
525                 530                 535                 540

caa acc aat aaa aag att ctc att gga aag tct gat gtt cat gga tgg   1681
Gln Thr Asn Lys Lys Ile Leu Ile Gly Lys Ser Asp Val His Gly Trp
                545                 550                 555

ggt gca ttt aca tgg gac tct ctt aaa aag aat gag tat ctc gga gaa   1729
Gly Ala Phe Thr Trp Asp Ser Leu Lys Lys Asn Glu Tyr Leu Gly Glu
            560                 565                 570

tat act gga gaa ctg atc act cat gat gaa gct aat gag cgt ggg aga   1777
Tyr Thr Gly Glu Leu Ile Thr His Asp Glu Ala Asn Glu Arg Gly Arg
        575                 580                 585

ata gaa gat cgg att ggt tct tcc tac ctc ttt acc ttg aat gat cag   1825
Ile Glu Asp Arg Ile Gly Ser Ser Tyr Leu Phe Thr Leu Asn Asp Gln
    590                 595                 600

ctc gaa atc gat gct cgc cgt aaa gga aac gag ttc aaa ttt ctc aat   1873
Leu Glu Ile Asp Ala Arg Arg Lys Gly Asn Glu Phe Lys Phe Leu Asn
605                 610                 615                 620

cac tca gca aga cct aac tgc tac gcc aag ttg atg att gtg aga gga   1921
His Ser Ala Arg Pro Asn Cys Tyr Ala Lys Leu Met Ile Val Arg Gly
                625                 630                 635

gat cag agg att ggt cta ttt gcg gag aga gca atc gaa gaa ggt gag   1969
Asp Gln Arg Ile Gly Leu Phe Ala Glu Arg Ala Ile Glu Glu Gly Glu
            640                 645                 650

gag ctt ttc ttc gac tac tgc tat gga cca gaa cat gcg gat tgg tcg   2017
Glu Leu Phe Phe Asp Tyr Cys Tyr Gly Pro Glu His Ala Asp Trp Ser
        655                 660                 665

cgt ggt cga gaa cct aga aag act ggt gct tct aaa agg tct aag gaa   2065
Arg Gly Arg Glu Pro Arg Lys Thr Gly Ala Ser Lys Arg Ser Lys Glu
    670                 675                 680

gcc cgt cca gct cgt tag tttttgatct gaggagaagc agcaattcaa          2113
```

Fig. 1c

```
Ala Arg Pro Ala Arg
685                 690

gcagtccttt ttttatgtta tggtatatca attaataatg taatgctatt ttgtgttact 2173

aaaccaaaac ttaagtttct gttttatttg ttttagggtg ttttgtttgt atcatatgtg 2233

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aaaaaaaaaa aaaaaactcg ag                                          2315
```
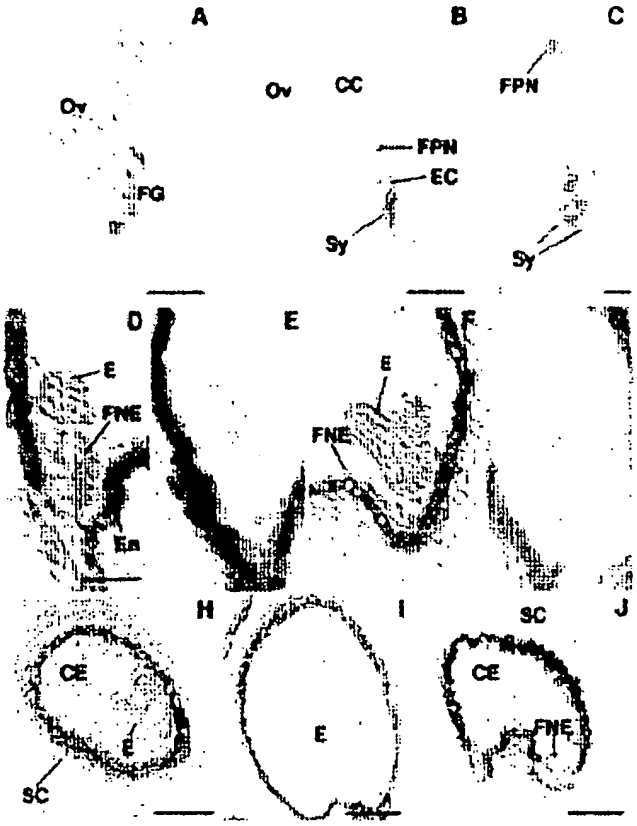

Fig. 1d


A
B
C
Ov
CC
FPN
Ov
FPN
EC
FG
Sy
Sy
D
E
F
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FNE
E
FNE
Ep
H
I
SC
J
CE
E
CE
E
FNE
SC

Fig.2

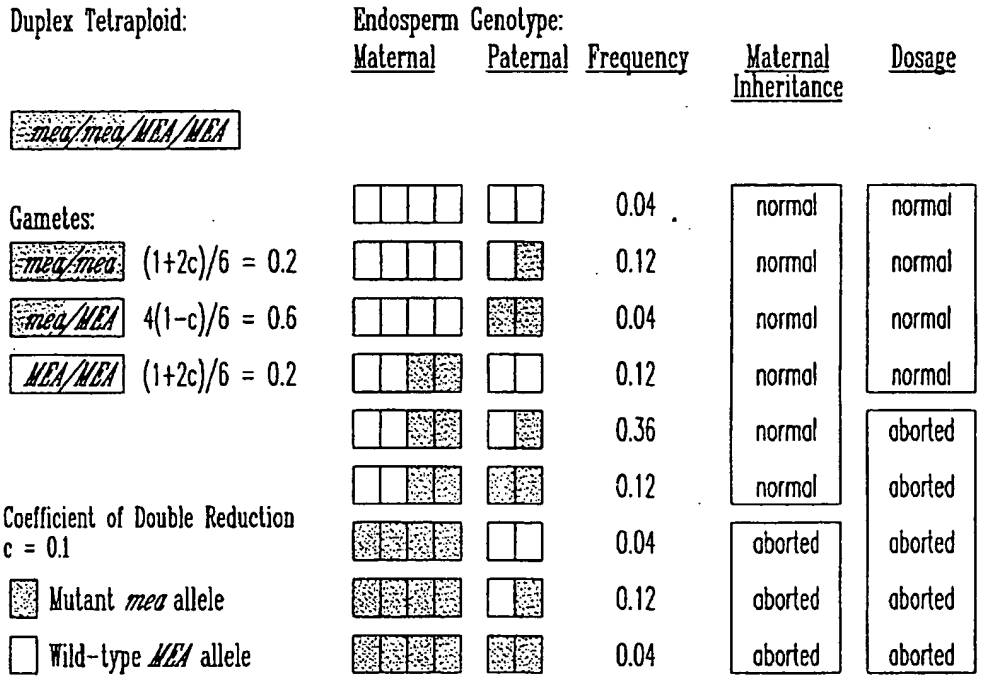
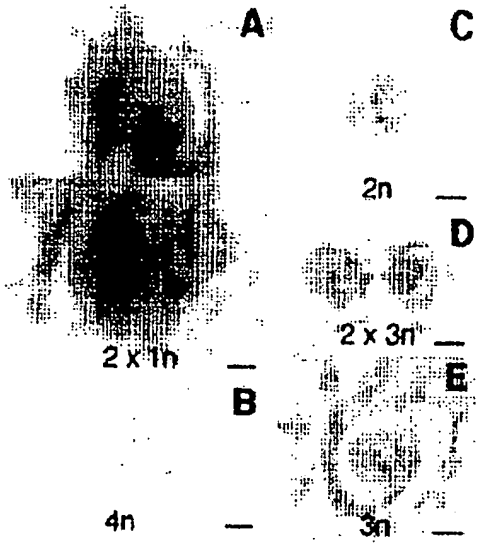

Duplex Tetraploid:
mea/mea/MEA/MEA
Gametes:
mea/mea (1+2c)/6 = 0.2
mea/MEA 4(1-c)/6 = 0.6
MEA/MEA (1+2c)/6 = 0.2
Coefficient of Double Reduction
c = 0.1
Mutant mea allele
Wild-type MEA allele
Endosperm Genotype:
Maternal
Paternal
Frequency
Maternal Inheritance
Dosage
0.04 normal normal
0.12 normal normal
0.04 normal normal
0.12 normal normal
0.36 normal aborted
0.12 normal aborted
0.04 aborted aborted
0.12 aborted aborted
0.04 aborted aborted

Fig. 3


A
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D
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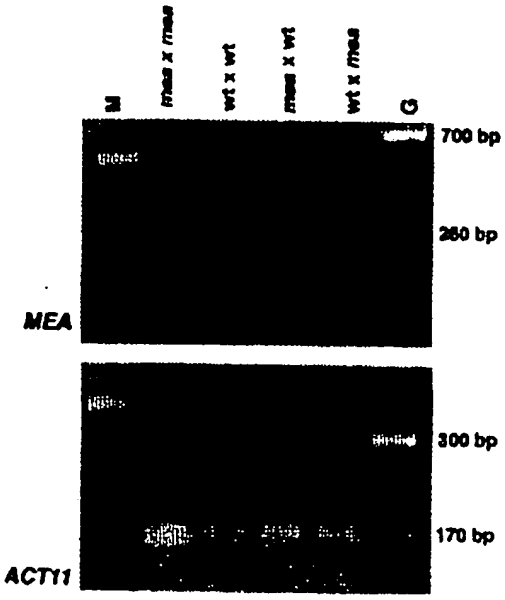
2 x 1n
E
B
4n
3n

Fig. 4


M
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wt x wt
mea x wt
wt x mea
G
700 bp
260 bp
MEA
300 bp
170 bp
ACT11

Fig. 5

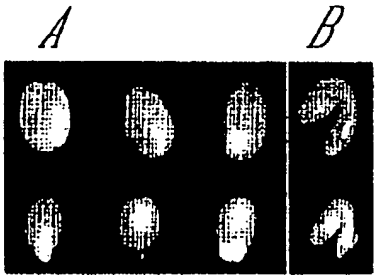

Fig. 6

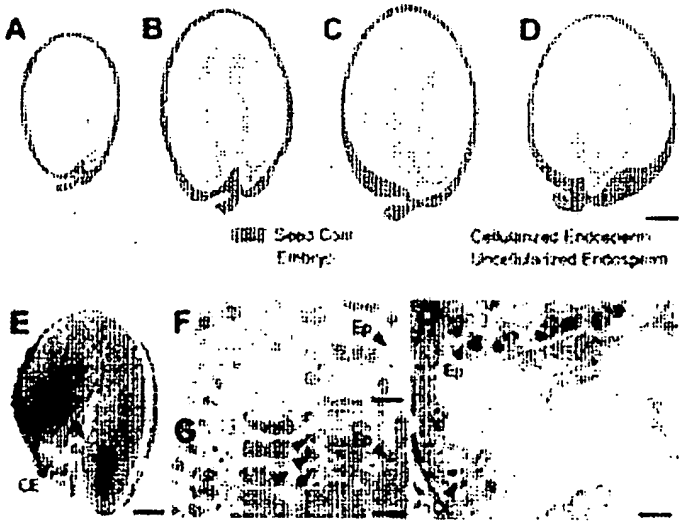

Fig. 7

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 00/31428

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C07K14/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VIELLE-CALZADA JEAN-PHILIPPE ET AL: "Maintenance of genomic imprinting at the Arabidopsis medea locus requires zygotic DDM1 activity." GENES & DEVELOPMENT, vol. 13, no. 22, 15 November 1999 (1999-11-15), pages 2971-2982, XP002164463 ISSN: 0890-9369 the whole document	1-26
Y	WO 99 53083 A (GROSSNIKLAUS UELI ;COLD SPRING HARBOR LAB (US); VIELLE CALZADA JEA) 21 October 1999 (1999-10-21) page 11, line 29 -page 12, line 3; claims 14-23,28-34	1-26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 April 2001	25/04/2001

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Internat al Application No
PCT/US 00/31428

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GROSSNIKLAUS ET AL: "maternal control of embryogenesis by MEDEA, a Polycomb group gene in Arabidopsis" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 280, 17 April 1998 (1998-04-17), pages 446-450, XP002111702 ISSN: 0036-8075 cited in the application the whole document	1-26
A	KAKUTANI T ET AL: "CHARACTERIZATION OF AN ARABIDOPSIS THALIANA DNA HYPOMETHYLATION MUTANT" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 23, no. 1, 1995, pages 130-137, XP002049118 ISSN: 0305-1048 the whole document	1-26
A	GOODRICH JUSTIN: "Plant development: Medea's maternal instinct." CURRENT BIOLOGY, vol. 8, no. 14, 2 July 1998 (1998-07-02), pages R480-R484, XP000985937 ISSN: 0960-9822 the whole document	1-26
A	WO 99 57247 A (OHAD NIR ;KIYOSUE TOMOHIRO (JP); GOLDBERG ROBERT B (US); HARADA JO) 11 November 1999 (1999-11-11) the whole document	1-26

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. ial Application No
PCT/US 00/31428

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9953083 A	21-10-1999	AU 3563199 A	01-11-1999
		EP 1071799 A	31-01-2001
WO 9957247 A	11-11-1999	AU 3783399 A	23-11-1999
		EP 1073718 A	07-02-2001